Do they feel the heat –

the impact of rising water temperatures on the bacterial community composition during algal springbloom conditions

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Katja Walther

aus Esslingen am Neckar

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Erstgutachter: Prof. Dr. Klaus Jürgens (Institut für Ostseeforschung Warnemünde, Rostock/Warnemünde)

Zweitgutachter: PD Dr. Thorsten Brinkhoff (Insititut für Chemie und Biologie des Meeres; Oldenburg)

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List of abbreviations

BCC	bacterial cell counts
CARD-FISH	catalyzed reporter deposition-FISH
Chl a	Chlorophyll a
CO2	Kohlendioxid
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4',6'-Diamidino-2-phenylindol Hydrochlorid
DGGE	Denaturierende Gradienten Gelelektrophorese
DNA	desoxy ribonucleic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
et al.	et alii
FISH	fluorescence in situ hybridization
ml	Milliliter
N	nitrogen
NCBI	National Center for Biotechnology Information
NA	non-attached
P	phosphorus
PA	particle-attached
PCR	polymerase chain reaction
POC	particulate organic carbon
rRNA	ribosomal ribonucleid acid
TBN	total bacterial numbers
thy	thymidine

VLP virus-like particles

Abstract

Future rising temperatures due to global warming are expected to influence marine life on all trophic levels. The assumed effect on microbial organisms is of particular interest because of their relevance for the marine carbon cycle. Especially the functional coupling between phyto- and bacterioplankton plays a major role in the fate of carbon in the world oceans. The importance of this link for the marine carbon cycle becomes even clearer when considering that an enhanced increase in temperatures is predicted for the early months of the year when fixation of atmospheric CO_2 is intensified by occurring phytoplankton spring blooms.

Within the DFG funded priority project AQUASHIFT, the work of the Kiel mesocosm cluster aimed at shedding light on the impact of rising water temperatures on the planktonic food web. Proceeding on the assumption that their different Q_{10}-factors will involve different effects of increasing temperatures on autotrophic and heterotrophic organisms, the functional mechanisms within the planktonic community in terms of match-mismatch und coupling-decoupling were analysed. Within this scope, the present study investigated possible temperature-related impacts on the bacterial community structure in particular, always against the backdrop of the above mentioned functional coupling between autotrophic marine algae and heterotrophic bacteria.

During indoor mesocosm experiments designed to simulate algal spring blooms over a temperature gradient, the bacterial community was sampled daily to analyse quantitative as well as qualitative bacterial dynamics. Furthermore, samples were analysed to monitor top-down controlling factors like viruses and heterotrophic nanoflagellates as well as possible bottom-up effects from organic and inorganic nutrients. Also, data to observe the development of highly active bacteria were obtained. The not-attached as well as the particle-attached fraction of bacterial assemblage were analysed on a molecular level using the genetic fingerprint method DGGE (denaturing gradient gel electrophoresis), followed by sequencing and identification of dominant excised bands. For the assessment of the group specific structure of the bacterial community, the highly sensitive CARD-FISH method was used. For all conducted experiments, data on physicochemical parameters like temperature, salinity, chlorophyll *a* and nutrient concentrations, were provided by members of fellow projects on AQUASHIFT.

The major results of this thesis can be summed up as follows:

- The experimental set up allowed a successful simulation of the development of a planktonic community during an algal spring bloom along a temperature gradient. The phytoplankton spring bloom itself could be effectively initiated, along with the expected successions within the planktonic communities as well. Though it is certainly not possible to directly transfer the results obtained within the Kiel mesocosm cluster to *in-situ* conditions, we therefore assume that the analyses that have been carried out allow a basic description of the mechanisms defining the functional relationships in terms of coupling-decoupling between the present planktonic communities.
- Among the bacterial community, highly active cells exhibited clearly different quantitative developments at different ambient temperatures, the dynamics indicating distinct grazing pressure at high temperatures which markedly decreased in cold mesocosms. Since it was not possible to link this effect to according grazer counts and therefore a possibly mediated temperature affect, it remains unclear whether temperature exerted a direct or rather indirect influence on the highly active bacterial community.
- The community structure of the present bacterial assemblage seemed to be directly affected by increasing ambient temperatures. Both the DGGE fingerprints as well as the quantitative CARD-FISH analysis showed temperature-related changes within the structure of the bacterial assemblage which, though detectable in both the not-attached and the particle-attached fraction, were more prominent in the latter.
- Different nutrient conditions seemed to have an impact on the temperature effect on the bacterial community structure. At high N-concentrations, the effect temperature exerted on the bacterial community composition apparently was intensified, though the particle-attached fraction still clearly was more affected. Nutrient conditions in the oceans should therefore be considered for a realistic assessment of the effect of rising water temperatures on the bacterial community composition.

Kurzzusammenfassung

Der künftige Anstieg der Umgebungstemperaturen aufgrund der globalen Erwärmung wird das Leben in den Meeren voraussichtlich auf allen trophischen Ebenen beeinflussen. Wegen ihrer entscheidenden Rolle innerhalb des marinen Kohlenstoffkreislaufs ist der vermutete Effekt erhöhter Wassertemperaturen auf mikrobielle Organismen hierbei von besonderem Interesse. Vor allem die funktionelle Kopplung zwischen marinem Phyto- und Bakterioplankton ist von besonderer Relevanz für den Verbleib von Kohlenstoff in den Ozeanen. Wird in Betracht gezogen, dass die vorhergesagte Erwärmung insbesondere die ersten Monate des Jahres und damit die Phase erhöhter Fixierung von atmosphärischem CO_2 während der Algenfrühjahrsblüten betreffen soll, wird die Bedeutung der engen Verbindung zwischen Algen und Bakterien umso deutlicher.

Die Arbeit des Kieler Mesokosmos-Clusters innerhalb des von der DFG geförderten Schwerpunktprogramms AQUASHIFT hatte daher zum Ziel, den Einfluss steigender Wassertemperaturen auf die funktionellen Mechanismen hinter dem planktonischen Nahrungsnetz zu klären. Vor dem Hintergrund der Annahme, dass steigende Temperaturen aufgrund unterschiedlicher Q_{10}-Werte unterschiedliche Auswirkungen auf heterotrophe und autotrophe Organismen haben, wurden die funktionellen Mechanismen innerhalb der Planktongemeinschaft hinsichtlich der Frage nach match-mismatch und Kopplung-Entkopplung untersucht. Im Rahmen dieser Zielsetzung wurde die vorliegende Arbeit durchgeführt, deren Schwerpunkt auf der oben genannten Kopplung zwischen marinem Phyto- und Bakterioplankton und dabei hauptsächlich auf der Untersuchung möglicher temperaturbedingter Verschiebungen in der bakteriellen Gemeinschaftsstruktur lag.

Mesokosmosexperimente, die in Klimakammern durchgeführt wurden, dienten zur Simulation von Algenfrühjahrsblüten unter dem Einfluss eines Temperaturgradienten. Um die quantitative wie auch die qualitative Entwicklung des vorhandenen Bakterioplanktons zu untersuchen, wurde die bakterielle Gemeinschaft während dieser experimentellen Algenblüten täglich beprobt und anschließend mit molekularbiologischen Methoden analysiert. Weiterhin wurde die Dynamik von zwei top-down Kontrollfaktoren des Bakterioplankton (virus-ähnliche Partikel und Nanoflagellaten) untersucht, ebenso wie organische und anorganische Nährstoffe als mögliche bottom-up Faktoren. Weiterhin fand eine quantitative Erhebung der hochaktiven Bakterien statt. Zur molekularbiologischen Untersuchung der nicht-angehefteten

wie auch der Partikel-angehefteten Bakterienfraktion kam die genetische fingerprint Methode DGGE (**d**enaturating **g**radient **g**el **e**lectrophoresis) zum Einsatz. Hierbei ausgeschnittene dominante Banden wurden sequenziert und identifiziert. Die gruppenspezifische Analyse der Bakteriengemeinschaft wurde mithilfe der hochsensiblen CARD-FISH Methode durchgeführt. Für die Analyse aller durchgeführten Experimente wurden Daten zu physikalisch-chemischen Parametern wie Temperatur, Salinität, Chlorophyll *a* und Nährstoffkonzentrationen von Mitgliedern aus Partnerprojekten innerhalb von AQUASHIFT zur Verfügung gestellt.

Die wesentlichen Resultate dieser Arbeit können folgendermaßen zusammengefasst werden.

- Das Design der durchgeführten Experimente ermöglichte eine erfolgreiche Simulation der Entwicklung der Planktongemeinschaft während einer Algenfrühjahrsblüte entlang eines Temperaturgradienten. Der Einsatz der Algenblüte selbst konnte in allen Fällen erfolgreich stimuliert werden, ebenso die erwarteten Sukzessionen innerhalb der Planktongemeinschaft. Ein direkter Übertrag der hier vorgelegten Ergebnisse auf natürliche Bedingungen ist sicherlich nicht möglich; die Analysen ermöglichen aber eine grundlegende Beschreibung der Mechanismen der funktionellen Verbindung innerhalb der Planktongemeinschaft hinsichtlich Kopplung-Entkopplung.
- Innerhalb der Bakteriengemeinschaft zeigten hochaktive Zellen deutlich unterschiedliche quantitative Entwicklungen bei unterschiedlichen Temperaturen. Die Verläufe der entsprechenden Zellzahlen deuteten auf höheren Fraßdruck durch heterotrophe Nanoflagellaten hin, der mit abnehmenden Temperaturen klar nachzulassen schien. Da dieser Effekt nicht mit entsprechenden quantitativen Entwicklungen der Nanoflagellaten und damit einem möglichen vermittelten Temperatureffekt in Verbindung gebracht werden konnte, konnte nicht geklärt werden, ob die hochaktive bakterielle Gemeinschaft durch steigende Temperaturen beeinflusst wurde – und wenn ja, ob es sich um einen direkten oder indirekten Effekt handelte.
- Die Zusammensetzung der untersuchten bakteriellen Gemeinschaften schien direkt durch steigende Temperaturen beeinflusst zu sein. Auf phylogenetischer wie auch auf gruppenspezifischer Ebene wurden durch DGGE fingerprints und die quantitative CARD-FISH Analyse Veränderungen in der Struktur der Bakteriengemeinschaft nachgewiesen, die möglicherweise mit der Temperatur in Verbindung standen.

Obwohl diese Verschiebungen in beiden Fraktionen auftraten, waren sie deutlich klarer in der Partikel-angehefteten Fraktion zu erkennen.

- Mit unterschiedlichen Nährstoffkonzentrationen schien sich auch der Temperatureffekt auf die bakterielle Gemeinschaftsstruktur zu ändern. Auch der Einfluss der Temperatur auf die Gemeinschaftszusammensetzung schien bei hohen N-Konzentrationen intensiver zu werden, hatte aber nach wie vor größere Auswirkung auf die Partikel-assoziierte Fraktion. Zu einer realistischen Abschätzung der Auswirkungen steigender Wassertemperaturen auf die Zusammensetzung der marinen Bakteriengemeinschaften sollten daher die herrschenden Nährstoffsituationen in den Ozeanen nicht außer Acht gelassen werden.

Introduction

Within the scope of the general global warming that has been predicted for the recent century by the IPCC (Houghton et al. 2001) due to continually released greenhouse gases, the temperatures of the Baltic Sea are expected to increase even more than the global mean (BALTEX report 2006). Already during the past century, the mean temperature increase in the Baltic region exceeded that of the global mean by 0.2 °C. Though this development could not be reliably ascribed to human influence and was yet within the range of natural variability, the described increase in temperature is in line with the climate predictions made by climate models working with increasing levels of atmospheric greenhouse gases.

The increased warming in the Baltic region compared to that taking place globally is strongest and most even in spring, with the winter effect still being stronger than that in summer and autumn (Climate Change in the Baltic Sea Area – HELCOM thematic assessment in 2007). As a consequence, climatic spring season starts earlier and the number of cold nights diminishes whereas that of warm days increases. The main reason for the Baltic being among those areas more affected by the globally increasing temperatures might be its exposition to certain atmospheric circulations: The overall winter surface climate in northern Europe has been strongly influenced by an intensifying westerly airflow during wintertime in the 1990s, which, for example, resulted in higher winter temperatures as well as precipitation and wind speed (Climate Change in the Baltic Sea Area – HELCOM thematic assessment 2007).

These atmospheric givens notwithstanding, it is still the increasing concentrations of greenhouse gases, which are predicted to result in a substantive increase of temperatures and the respective climate change within this century. The expected rise in temperatures ranges between 1.4 and 5.8°C (Cubasch et al. 2001), factoring in different scenarios of CO_2 emissions and discrepancies between several climate models. The trend of an intensified warming of the Baltic region during the past century, however, constantly remains in further predictions of the future climate by models. Regional climate models based on a GCM (**g**lobal **c**limate **m**odel) predict rising near surface air temperatures over the Baltic Sea. Compared with summer scenarios, winter temperatures show a stronger increase in northern and eastern regions of the Baltic Sea in these simulations (Climate Change in the Baltic Sea Area - HELCOM thematic assessment 2007). The IPCC report of 2001 predicted an increase in annual mean surface temperature of 3 to 5°C for the “business as usual scenario” (doubling of CO_2 - emissions during the 21st century) for the Baltic Sea region. Compared with the reference period from 1960-1990, the “business as usual scenario” prognosticates winter temperatures for the years 2070-2100 that might even increase by 5-10°C.

This change in ambient and therefore also in water temperatures is assumed to affect biota in the Baltic Sea, especially during the winter and early spring months, which is the major vegetation period of the year. Not only are the predicted changes thought to affect macro fauna like water birds and their mortality and migration behavior, or the production and survival rates of fish like sprat and herring (Climate Change in the Baltic Sea Area - HELCOM thematic assessment 2007). Temperature shifts are also expected to have an impact on smaller organisms, such as wintertime survival of overwintering zooplankton species (HELCOM thematic assessment 2007). Furthermore, phytoplankton as the major primary producers of organic matter in the Baltic Sea are assumed to be affected as well. As a result, algal bloom composition might change with warmer winters until the diatoms usually dominating the phytoplankton spring blooms might be replaced by dinoflagellates (HELCOM thematic assessment 2007). But also smaller organisms directly consuming algal derived organic matter might be affected by increasing winter/spring water temperatures. Heterotrophic bacteria attached to particles and aggregates as well as those living freely suspended in the water column might be stimulated in their activity, which is assumed to lead to a mismatch between phytoplankton production on the one and bacterial consumption of algal-derived organic matter on the other hand.

Marine algae and bacteria – let's stick together

In the photic zone of the world's oceans, primary production by phytoplankton rivals that of terrestrial primary production, with an estimated global net primary production of 45-50 Gt per year (Longhurst et al. 1995). A rather small amount of this marine carbon is exported to the oceans' depths in form of particles and aggregates; about 95% of the marine organic matter is available as dissolved organic carbon (DOC), which is assumed to be mainly decomposed by heterotrophic bacteria (Fenchel & Blackburn 1979, Wetzel 1982). As the essential members of the 'microbial loop' (Azam et al. 1983), these bacteria convert DOC into particulate organic matter, a process by which, at the same time, inorganic nutrients are released. Both these minerals as well as organic carbon in its particulate form serve as fuel for the marine food web on higher trophic levels. With about 50% of the marine primary production being channeled through the microbial loop (Hagström et al. 1988), the relevance of heterotrophic bacterial secondary production for the marine carbon as well as the biogeochemical cycle of inorganic nutrients becomes explicit.

During the past decades, several studies suggested that bacterial production is strongly linked to chlorophyll *a* as well as primary production (Simon and Tilzer 1987, Cole et al. 1988, Kirchman et al. 1995). Dissolved organic matter released by phytoplankton in form of exudates during algal growth and phytoplankton decomposition apparently sustains secondary production by the bacterial community living freely suspended in the water column (Reimann et al. 1982, Larsson and Hagström 1982, Sell and Overbeck 1992). Furthermore, also the particulate fraction of the marine organic matter in form of aggregates and organic particles is utilized by the bacterial assemblage capable of attaching itself to the surfaces of said particles, which mainly form during and after their degradation phase of phytoplankton blooms (Kirchman 1983). Apart from this obvious close coupling between algal primary production on the one and not-attached as well as particle-attached bacterial activity on the other hand, bacterial biomass itself responds to other limiting or regulating factors. Inorganic nutrient concentrations (Horrigan et al. 1988, Heinänen and Kuparinen 1992), grazing pressure by heterotrophic protists (Wright and Coffin 1984, Weinbauer and Höfle 1998) as well as cell lysis caused by viral infection (Weinbauer and Höfle 1998) strongly influence bacterial standing stock in the oceans, even though their extents vary depending on the trophic character of the respective system. In areas with high productivity such as coastal regions with strong up-welling events or systems, bacterial biomass is assumed to be limited by top-down effects like grazing and viral lysis. In systems with low productivity, though, for example in the cold oceans of the Arctic and Antarctic, the main limiting factors for numerical bacterial development apparently are dissolved organic carbon along with mineral resources (Church et al. 2000). Yet there is also indication that there is a combined effect of several factors on bacterial development, including temperature, organic and inorganic nutrients (Wiebe et al. 1993, Pomeroy et al. 1993, Church et al. 2000).

Phytoplankton is the main source of dissolved organic material (DOM). DOM ‘leaks’ into the surrounding water during the algal bloom itself (Bjørnsen, 1988), but also the degradation phase of a phytoplankton bloom involves polymer hydrolysis by attached bacteria and therefore the release of organic material (Chróst et al. 1989, Middelboe et al. 1995). Particulate remnants of phytoplankton cells are degraded by heterotrophic bacteria and thus channeled back into the pool of DOM.

Dissolved organic carbon (DOC), though not completely identified yet, mainly consists of carbohydrates, fatty acids and amino acids, all of which are important food resources for marine heterotrophic bacteria (Wheeler and Kirchmann 1986, Kirchman and Ducklow 1987, Keil and Kirchman 1999). Most of these compounds cannot be immediately utilized by

bacteria since they are polymeric and therefore too large. After having been hydrolyzed exoenzymatically, polysaccharides, on the other hand, can be directly assimilated.

The other organic carbon pool exploited by heterotrophic bacteria consists of particular organic matter (POM). Organic carbon is integrated in aggregates and particles in varying proportions in the form of carbohydrates and amino acids, depending on the origin of the particulate matter (Azam and Cho, 1987; Smith et al.1995; Biddanda & Benner 1997). The concentration of heterotrophic bacteria attached to POM is much higher than in the surrounding water (Simon et al. 2002), which is a clear indication for the bacterial utilization of POM. The break-down of macromolecules by hydrolytic exoenzymes (Hoppe et al. 1988; Madigan, 2003) results in the formation of DOC, which is utilized again by freely suspended as well as particle-attached heterotrophic bacteria. This way of recycling organic nutrients from senescent phytoplankton material, which can again be channeled to higher trophic levels is called 'the microbial loop' (Azam et al. 1983).

Organic material provided by algal primary production is therefore the main source of substances utilized by bacteria. DOC directly released during the bloom or its break down is immediately taken up by the not-attached community; POC is first degraded by particle-attached bacteria to smaller molecules, which finally contribute to the DOC pool as well. Bacteria are therefore highly dependent on algal primary production which is equivalent with direct or indirect DOC supply. We refer to this close functional relationship between phyto- and bacterioplankton as 'coupling', whose intensity depends on several factors. Moran and colleagues (2002) found that different marine systems apparently select for algae-bacteria couplings of different extent. This is not surprising, given the fact that the strength of the link between phyto- and bacterioplankton depends on the relevance of autochthonous production for a given system as well as the factors limiting bacterial activity. Aside from the above mentioned 'top-down' factors, there are also the limitations working the other way around: 'bottom-up', among them geochemical limitation by inorganic nutrients such as nitrate, phosphate or iron, but also physical factors like the one being a major subject of this thesis – ambient water temperatures.

As it is to be expected for heterotrophic organisms, higher ambient temperatures apparently result in increased bacterial activity (White et al.,1991; Shiah & Ducklow, 1994). Autotrophic organisms like phytoplankton, however, are primarily light dependent with temperature only playing a secondary role (Tilzer et al. 1986). The Q_{10} factors (describing the factorial increase in any rate at an increase of 10°C in ambient temperature) of both bacteria and phytoplankton

illustrate this notion: whereas algae primary production usually exhibits a Q_{10} factor between 1 and 2 and a clear dependence from light conditions (Tilzer et al. 1986), the typical Q_{10} factor of bacterioplankton activity which ranges between 2 and 3 (Pomeroy & Wiebe 2001) shows that bacteria are more effectively determined by ambient temperatures than phytoplankton is. This does not only imply that, given the right light conditions and translucence of the water body, algal blooms can occur at very low temperatures without a corresponding bacterial development. It also entails that, provided that temperatures allow parallel phyto- and bacterioplankton growth in the first place, bacterial consumption of organic matter does not match the respective algal DOC and POC production at low ambient temperature.

These considerations notwithstanding, it has been shown that high bacterial activity can be found even in very cold environments. Not only were heterotrophic bacteria in sea ice and polar seas apparently not inevitably limited at temperatures below freezing point (Robinson & Williams, 1993; Rivkin et al. 1996), but their development also followed phytoplankton dynamics (Yager et al. 2001). Evidently, low temperatures do not necessarily have to result in a de-coupling of algal production and bacterial consumption. The above mentioned studies might indicate that the bacterial demand for substrate, which is assumed to constantly grow with increasing cold (Nedwell, 1999), is met by the algal production during the arctic phytoplankton blooms. This pulse in organic substrate thus enables the bacteria to overcome the initial temperature induced inhibition (Nedwell & Rutter 1994; Pomeroy & Wiebe 2001). Another possible explanation, however, points to the composition of the bacterial community.

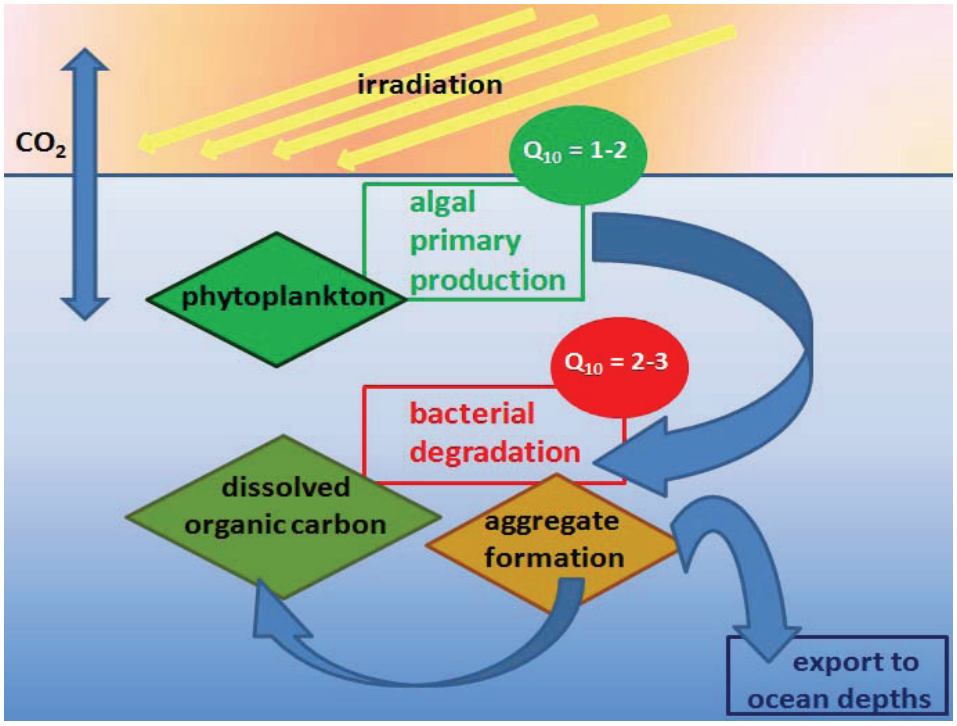


Fig.1: Temperature dependence as represented by Q_{10} values of the interaction between phytoplankton production and bacterial degradation

The phylogenetic structure of a given bacterial assemblage is subject to several impacts and influences. The organic substrate utilized by bacteria selects for certain specific taxa, depending on complexity and character of the present organic molecules (Cotrell & Kirchman 2000). The specific organic matrix of phytoplankton exudates has also been shown to be a factor of reasonable influence on the bacterial community composition (Pinhassi et al. 2004, Grossart et al. 2005). Furthermore, bacterial assemblages attached to particles are different from those living freely suspended in the water column (Fandino et al. 2001, Simon et al. 2002, Grossart et al. 2005). Regarding the unexpected highly active bacterial communities found in arctic waters and sea ice, it seems like temperature might be another factor regulating the structure of a given bacterial community.

The results of several studies carried through in mesocosms as well as *in-situ* conditions during the past years indicate that changes in bacterial community composition go hand in hand with shifts in the metabolic properties of an observed bacterial assemblage (Riemann et al. 2000, Fandino et al. 2001; Yager et al. 2001). Though these works focused on variations occurring during the different stages of a proceeding algal bloom, they imply that a shift in ambient conditions and therefore a change in demands for bacterial degradation properties, select for a particular bacterial community capable of meeting those requirements.

Aside from the above mentioned quantity and quality of organic carbon itself, there are further environmental influences ("bottom-up" factors) potentially affecting bacterial degradation, like inorganic nutrient concentrations. The availability of inorganic F and P as well as organic N, for example, has been found to influence marine bacterial growth (Kirchman 1990, Rivkin & Anderson 1997, Sala et al. 2002, Thingstad et al. 2005). Furthermore, it has been shown that not only bacterial activity but also the community structure of a bacterial assemblage can be affected by differing inorganic nutrient conditions (Hutchins et al. 2001, Pinhassi & Berman 2003, Pinhassi et al. 2006).

Like inorganic nutrients, ambient temperature is assumed to affect both bacterial degradation activity as well as community structure. It is easily conceivable that phytoplankton blooms taking place at colder temperatures are likely to harbor bacteria with lower activity optima than those occurring at warmer ambient conditions. Following this notion, a shift in bacterial community composition from cold-adapted assemblages to those with higher activity optima would be a logical consequence of rising water temperatures due to global warming. Considering that such shifts within the bacterial community composition might come along with changes of their respective degrading properties, it becomes clear that increasing ambient temperatures especially during phytoplankton spring blooms might be of relevance for the decomposition of the algal derived organic matter. Different dominating bacterial phylotypes might also involve different bacterial enzymatic features (Martinez et al. 1996), which eventually could affect the microbial processing of organic matter and therefore the marine carbon cycle as a whole. Hence, for being able to appraise the effect of global warming on the bacterial role in the processing of marine organic matter, it is not enough to learn about how rising ambient temperatures during phytoplankton blooms affect bacterial activity, but it is necessary as well to improve the knowledge about the impact of increasingly warm waters on the bacterial community composition.

Considering the fact that ambient water temperature as well as inorganic nutrient concentrations both have an impact on bacterial activity as well as bacterial community composition, it might be necessary to take both factors into account for a realistic assessment of the impact of global warming on the functional coupling between phyto- and bacterioplankton and therefore the marine carbon cycle. The evidence for seasonally varying inorganic nutrient conditions as well as different sea areas revealing different primary limiting inorganic nutrients (Sala et al. 2002, Pinhassi at al. 2006) is suggesting that the actual impact of increasing water temperatures can only be appropriately described by taking the inorganic nutrient conditions into account.

Bacterial communities and their composition

Prior to the introduction of molecular biological methods to microbial ecology, culture-dependent methods and microscopy were the sole options for the analysis of bacterial community compositions. As it turned out, both kinds of methods exhibited certain shortcomings: culture-dependent methods did not allow cultivating bacteria under natural conditions; under artificial conditions some species are easier to grow than others, with some bacteria not being cultivatable at all. This in combination with the severely restricted option to morphologically identify bacteria under the microscope resulted in a long time underestimation of the biological diversity of marine bacteria. The possibility of comparing several genotypes, using the highly conservative 16S rRNA gene fragment, shed new light on bacterial community composition and microbial ecology (Woese et al. 1987). The most established molecular biological methods to analyse the structure of bacterial assemblages are the restriction fragment length polymorphism (RFLP), rDNA intergenic spacer analysis (RISA), Realtime PCR, denaturating gradient gel electrophoresis (DGGE, Muyzer et al. 1993) and fluorescence in situ hybridization (FISH) as well as catalysed reporter deposition-FISH (CARD-FISH), respectively.

The screening of marine bacteria with the above mentioned methods revealed differences in their community structure with respect to their state of attachment (non-attached or particle-attached) as well as habitat. In a variety of marine environments, particle associated bacteria are dominated by the ***C****ytophaga/****F****lavo****b****acteria* (CFB) cluster, together with γ-*Proteobacteria*. α-*Proteobacteria,* on the other hand, appear to only play a minor role on marine snow particles (DeLong et al. 1993, Ploug et al. 1999, Bidle & Azam 2001). In lakes, the community composition of bacteria on lake snow aggregates looks quite different. The assemblages appear to be dominated by β-*Proteobacteria,* with α-*Proteobacteria* being the second most abundant group (Weiss et al. 1996, Grossart & Simon 1998, Schweitzer et al. 2001), whereas γ-*Proteobacteria* seem to be negligible, at least on particles in Lake Constance (Brachvogel et al. 2001).

For Rivers and estuaries, there are indications for differences in the bacterial community composition between the freshwater, brackish and marine section as well as between the bacterial fractions themselves. The particle-attached (PA) community in the limnetic section apparently is dominated by the CFB cluster (Simon et al. 2002, Selje & Simon 2003), whose prevalence decreases with rising salinity (Selje & Simon 2003). The proportions of the three

subclasses of PA-*Proteobacteria* also change along a salinity-gradient: β-*Proteobacteria* show a dominance in freshwater environments which shifts towards α- and γ-*Proteobacteria* with increasing salinity (Glöckner et al.1999, Böckelmann et al. 2000, Brümmer et al. 2000, Bouvier & del Giorgio 2002, Simon et al. 2002). Not-attached bacterial communities typically are dominated by β-*Proteobacteria* in the freshwater section of estuaries, whereas α-*Proteobacteria* take over towards marine environments with γ-*Proteobacteria* and the CFB group being of lower significance (Bouvier & del Giorgio 2002).

Taken together, the bacterial community composition in aquatic systems apparently shifts with salinity. In estuaries and brackish conditions like in the Kiel Bight, this shift tends to be a continuum from the freshwater towards the marine section, albeit distinct community structures can form along the salinity gradient. Irrespective of salinity, however, the non-attached and particle-attached aquatic bacterial fractions reveal obvious differences in community composition.

The Kiel Fjord as a part of the Baltic Sea – résumé of a model system

Having formed as a result of the retreat of the last Scandinavian ice-sheet about 12000 years ago, the Baltic Sea is a very young sea from a geological point of view (Köster 1996). Since water exchange with the adjacent North Sea is restricted to punctual inflow events and inflowing rivers represent the main water supply, the Baltic Sea is one of the world's biggest brackish water bodies (Rheinheimer 1996).

The fjords of the southern coast line of the Baltic Sea which developed when deep and narrow basins molded by glacier tongues or meltwaters were flooded during the warming of the climate, show a clear connection to the former glacial relief of this area (Lampe 1996). Compared to other marine areas, however, the depth of these Fjords, among them the Kiel Fjord from which the water for our mesocosm experiments was taken, is still moderate (Graf et al. 1983). This fact was of great importance for the choice of the Kiel Fjord as a model system for the Kiel mesocosm cluster within the priority program AQUASHIFT.

During early spring, the beginning of phytoplankton blooms which serve as the general background for our experiments is triggered by increasing light intensity which enables and stimulates algal primary production. Within deep marine or limnic areas, the murky homogenous water body as a result of winter mixing often inhibits sufficient light penetration, which leads to a delay of the start of the phytoplankton spring blooms. In these waters,

thermal stratification is required to keep the otherwise vertically circulating algae as close to the surface layer as possible for optimal exploitation of the improving spring light conditions.

The rather shallow brackish waters of the Kiel Fjord with a depth mostly less than 20 m (Babenerd and Gerlach 1987, Jochem 1989) present a quite different situation on several levels than it is typical for deep water bodies. Following the common paradigm of the *critical mixing depth concept* (Sverdrup 1953), thermal stratification is necessary for the formation of algal spring blooms in deep lakes or open ocean waters: the upper mixed layer needs to be shallower than a certain critical depth to provide light intensities favorable enough to trigger phytoplankton blooms (Mann and Lazier 1996, Obata et al. 1996). It is assumed that as a consequence to its shallow depth, thermal stratification is no necessary precondition for an early algal spring bloom in the Kiel Bight (Sommer et al 2007). Due to the variety of relatively shallow water bodies in North Germany like e.g. the Wadden Sea, the German Bight (both part of the North Sea) and numerous lakes, the water body from the Kiel Fjord, including its organisms as well as biogeochemical processes, can be regarded as a model system for a large part of marine as well as limnic water bodies in the North of Germany. Regarding the basic mechanisms of the interactions between phyto- and bacterioplankton, however, observations made in the Kiel Fjord during an algal spring bloom may also be considered representative for corresponding procedures during early phytoplankton blooms in the surface layer of deeper water bodies.

The AQUASHIFT Kiel mesocosm cluster

To be able to assess the general influence of increasing ambient temperatures on the bacterial community composition, this work was conducted within the Kiel mesocosm cluster of the DFG priority project AQUASHIFT.

For the investigation of community dynamics and ecological processes (the “part and the whole”, Odum 1984) at the same time, controlled experimental ecosystems have long been employed in aquatic ecology (Grice and Reeve 1982, Beyers and Odum 1993, Riemann et al. 2000, Fandino et al. 2005, Tanaka et al. 2008). Still, the use of mesocosms entails certain deficiencies which only allow a restricted transfer from the experimental system to *in-situ* conditions: for one thing the reduced size makes it difficult to cultivate organisms from higher trophic levels. Furthermore, the walls of mesocosms provide an artificial surface for biotic growth, for example benthic phyto- and bacterioplankton species (Chen et al. 1997).

Despite these difficulties, enclosed mesocosms allow the complete control of environmental parameters, for example of ambient temperature, as it was necessary in AQUASHIFT.

A total of eight indoor mesocosms was set up at four differently tempered climatic chambers of the IfM Geomar in Kiel (Germany), so each chamber hosted two parallel tanks at one defined ambient temperature. Light was supplied from above, the sophisticated regulation options allowing intensity and progression similar to *in-situ* conditions, but also more flexible settings if necessary. The tanks held a volume of approximately 1200 L and were manufactured of opaque polyethylene to reduce lateral irradiation and therefore minimize wall growth. With one propeller in each mesocosm, gentle and constant mixing of the water column was achieved in order to counteract sedimentation.

Four different temperature settings allowed the simulation of a temperature gradient. Based on the so called 'zero-treatment', which served as baseline and was calculated from *in-situ* average values over ten years in the sample area, the other three climatic chambers were set at 'zero-treatment' (Δ 0°C), +2 (Δ +2°C), +4 (Δ+4°C) and +6°C (Δ +6°C). Over the course of the experiment, temperatures were raised according to previous average calculations from *in-situ* temperatures.

The mesocosms were filled with unfiltered water from the Kiel Bight, including the overwintering plankton communities, which was first sampled in a collective tank and from there distributed evenly over eight hoses to provide similar water bodies in all mesocosms. Daily water samples were replaced with additional water from the Kiel Bight. The tanks were further stocked with zooplankton sampled in the Baltic to ensure *in-situ* concentrations, since the mortality among this community was high due to the filling process through hoses.

For appraising the possible combined effect of rising temperatures as well as inorganic nutrient concentrations, a different experimental set-up was used. Sticking to the initial AQUASHIFT principle of the artificial temperature gradient, a bottle experiment (bottle experiment summer 2007) was used to examine the algal-bacterial-coupling under different temperature and nutrient regimes. In indoor climate chambers, three temperature treatments were set up at +4°C, +8°C and +12°C. Water from 10 m depth at Bokins Eck (close to the mouth of the Kiel Fjord) was allowed to age in the dark for several weeks and afterwards filtered over 0.45 µm before it was filled into 25 L transparent nalgene bottles. At each temperature treatment, three bottles were set to the two different nutrient ratios N:P = 5 and N:P = 30. As inoculum, the remaining bacterial community as well as a pure axenic culture of

Skeletonema costatum was used and exposed to a light regime of 12:12 hours day-night-cycle. Water removed for sampling was not replaced.

Using these experimental set-ups, the formation of algal spring blooms was monitored at different ambient temperatures over the duration of several months. Table 1 shows the different settings of the experiments which have been sampled during the first period of the priority program AQUASHIFT (AQUASHIFT spring 2005 - AQUASHIFT spring 2007) as well as the bottle experiment summer 2007. The data presented in this study are the results of the analysis from the experiment AQUASHIFT spring 2006 and the bottle experiment.

Tab. 1: Overview of the designs of the two experiments conducted and analysed for the presented work

experiment	volume	organisms included	temperature gradient	additions	light intensity
AQUASHIFT spring 2005	~ 1200L	-overwintering planktonic community -zooplankton	Δ0°C – Δ6°C (in steps of 2°C)	_	- 30% max. intensity - in-situ progression
AQUASHIFT spring 2006	~ 1200L	-overwintering planktonic community -zooplankton	Δ0°C – Δ6°C (in steps of 2°C)	inorganic nutrients (to meet conditions of previous experiment)	-100% max. intensity -12:12 h day-night-cycle - even 6h in-/ decrease
AQUASHIFT spring 2007	~ 1200L	-overwintering planktonic community -zooplankton	Δ0°C – Δ6°C (in steps of 2°C)	_	- 60% max. intensity - in-situ progression
bottle experiment summer 2007*	~ 20L	-bacterioplankton -*Skeletonema costatum*	Δ4°C – Δ12°C (in steps of 4°C)	- nitrogen - phosphorus	-100% max. intensity -12:12 h day-night-cycle - even 6h in-/ decrease

*The bottle experiment carried through in summer 2007 was no 'official' AQUASHIFT experiment but conducted as a spin-off from the AQUASHIFT project.

The aim of the present work was to examine the response of the marine planktonic community during a pyhtoplankton spring bloom to the rising water temperatures due to future global warming. The main focus lay on potential shifts within the community composition of the bacterial assemblage by analysing both the not-attached and the particle-attached bacterial fractions. The assumed coupled effect of temperature and nutrient concentrations on the bacterial community structure was also taken into account. This study therefore provides valuable insights into the impact increasing water temperatures might have on the community composition of marine bacteria and therefore also on the processes defining the functional link between algal production and bacterial degradation of organic matter. Understanding the possible consequences of these experimental results for the *in-situ* planktonic communities will allow for a realistic appraisal of the impact of global warming on the marine carbon cycle.

A general overview of the development of the plankton community with increasing temperature is shown in chapter I "The effect of rising temperatures on a planktonic community during an induced phytoplankton spring bloom". Enumeration of phyto-, mesozoo-, bacterio- and viroplankton, the molecular analyses of the eu- and prokaryotic communities as well as the assessment of highly active bacteria indicated an impact of increasing temperature on the interaction between some of the different planktonic fractions. Within the bacterial assemblage, quantitative and apparently temperature-related shifts were most distinct in the highly active fraction of the not-attached bacterial community and most pronounced between the two temperature extremes (Δ 0°C and Δ +2°C). Though a direct impact of temperature could not be ruled out, these quantitative shifts were probably a result from indirect temperature effects, mediated by zooplankton predation. Still, these pronounced quantitative shifts within the highly active bacterial community made clear that, whether in a direct or indirect way, increasing temperature does effect the bacterial community. In the following chapters, this impact was analyzed more elaborately.

In this respect, chapter II "Temperature effects on the bacterial community composition during an algal spring bloom: a mesocosms study", describes the composition of the not-attached as well as the particle-attached bacterial community for the two extreme temperatures of the experiment AQUASHIFT spring 2006. With a focus on the molecular methods CARD-FISH and DGGE, the bacterial assemblage at Δ 0°C and Δ +6°C revealed quantitative and qualitative differences in community structure which were most pronounced in the PA fraction. The analysis shown in chapter III therefore revealed shifts within the

bacterial community during the experiment AQUASHIFT spring 2006 which appeared to be directly related to the ambient water temperatures.

Chapter III “Shifts in the bacterial community composition during an algal bloom under the influence of rising temperatures and different nutrient regimes” addresses the interplay between increasing temperatures and inorganic nutrients as another bottom-up factor to affect growth and activity of the bacterial community, The experimental design differed from that used in the AQUASHIFT experiments (table 1), still the same molecular methods were applied to examine the bacterial community structure along the temperature gradient of Δ 0°C, Δ +4°C and Δ +8°C. The results especially from the genetic fingerprinting DGGE revealed temperature linked structural differences within the bacterial assemblage, whose extent seemed to be influenced by different nutrient conditions.

Fig. 2: Climate chamber with mesocosm containing ~1200 L of sea water; devices for direct lighting from above in inclined position

I. The effects of rising temperatures on a planktonic community during an induced phytoplankton spring bloom

Abstract

An indoor mesocosms experiment was conducted to shed light on the influence of increasing water temperatures on the planktonic community during early phytoplankton spring bloom conditions. Over a temperature gradient of four different ambient temperatures, (Δ 0, Δ +2, Δ +4 and Δ+6°C from the in situ ten-year temperature average in the Kiel Fjord of 2°C), an assemblage of the natural winter plankton community from the Baltic was incubated and analysed during the course of an algal spring bloom. The planktonic community (phyto-, mesozoo-, bacterio- and viroplankton (virus-like particles) as well as heterotrophic nanoflagellates in general along with the abundance of actively respiring bacterial cells in particular was analysed by several staining methods, combined with different ways of microscopy and flow cytometry, respectively. Furthermore, the eukaryotic community as well as the not-attached bacterial assemblage were studied on a molecular level, using the PCR-based genetic fingerprint method denaturing gradient gel electrophoresis (DGGE), along with the sequencing of excised bands. The timing of the phytoplankton blooms was clearly influenced by ambient temperature. Quantitatively, algal dynamics and mesozooplankton abundance appeared to be linked in their quantitative development: higher over all-abundances occurred in the warmer tanks; furthermore, nauplii concentrations increased with rising temperatures. The banding patterns of the eukaryotic genetic fingerprint suggested differences in the structure of the eukaryotic communities at the two temperature extremes. Over the temperature gradient, the dynamics of heterotrophic nanoflagellates and virus-like particles could not be linked to different ambient temperatures. There were no qualitative temperature-related shifts within the not-attached bacterial community, but quantitative analysis revealed lowest total bacterial numbers at coldest temperatures. Highly active bacteria did show apparent temperature-related quantitative trends, revealing steep declines in their dynamics that varied with ambient temperature and could not be linked to temperature-dependent nanoflagellate activities or lysis events. Yet, since ciliates were not assessed in this study, grazing could not completely be ruled out as the cause for these temperature-linked dynamics of highly active bacterial cells.

Introduction

The fate of carbon in the oceans is determined by the functions of the marine food web whose structure is closely coupled with the cycling of organic matter (Thingstad and Rassoulzadegan 1999). Primary and secondary production of organic matter by phyto- and bacterioplankton and its mediation to higher trophic levels by grazing of nanoflagellates (mixo- and heterotrophic), ciliates and metazoa determines whether carbon is exported to be stored in sediments, whether it accumulates in the water column or re-enters exchange with the atmosphere. From primary production, bacteria mediate the major flux of organic matter to higher trophical levels via the microbial loop (Azam et al. 1983). Bacterioplankton is controlled by mixo- and heterotrophic protozoa, which in turn are preyed upon by ciliates. The grazing of metazoa on algae and ciliates is the mediating step from primary and secondary production to larger heterotrophs. The quantity as well as the quality of interaction between these 'executive' biota allows a description of the flux patterns for oceanic carbon. A thorough understanding of the marine (microbial) food web, its controlling agents and how they influence the fluxes of the marine carbon cycle is therefore crucial for a description of the role of the oceans within the global biogeochemical carbon flux (Legendre and Le Fevre, 1995).

With the set-in of a phytoplankton spring bloom event, phyto- and bacterioplankton as the constituent biota of the microbial food web undergo a temporal succession (Andersen & Sørensen 1986, Urban et. Al 1992, Larsen et al. 2004): primary production by algal growth sets off first, mainly triggered by sufficient light intensity (Hitchcock and Smayda 1977), which in turn enables the overwintered phytoplankton to develop, utilizing the inorganic nutrients that have accumulated in the water column during winter (Wasmund et al. 2006). Heterotrophic bacteria answer to the DOC released by phytoplankton with enhanced growth and enter into a competition for inorganic nutrients with phytoplankton (Bratbak 1987). In turn, heightened bacterioplankton activity results in an increase of bacterial grazers. Therefore, mixo- and heterotrophic nanoflagellates as well as flagellate-grazing ciliates follow the increasing bacterial numbers. Metazoans, which mainly prey on phytoplankton, also succeed the algal development and can influence the extent and duration of the spring bloom, depending on their overwintering stock (Bathman et al. 1990).

This close interaction of biota illustrates that the relative proportions of the functional components to each other play an important role for structure and performance of the general as well as the microbial food web and hence for the biogechemical cycling of marine carbon (Cho and Azam 1990). Nutrient concentrations seem to be a relevant issue where this interplay of planktonic biota is concerned. It has been shown that the ratio of the overall heterotrophic : autotrophic biomass in marine plankton communities as a proxy for their interaction varies. The share of heterotrophs apparently decreases with rising algal primary production, which has been associated with the trophic status of oceanic regions (Gasol et al. 1997). There is also evidence for bacterial biomass surpassing that of phytoplankton in oligotrophic conditions (Simon et al. 1992), whereas increasing trophic status favors autotrophic production (Gasol et al. 1997).

Aside from nutrients, temperature is a further regulative factor differently affecting heterotrophic and autotrophic plankton communities. This is shown by their respective Q_{10} values, which represent the factor a physiological rate has to multiplied with for an increase of 10°C in ambient temperature, and which tend to differ between heterotrophic and autotrophic plankton. The Q_{10} for phytoplankton is generally assumed to range from 1 to 2 (Eppley 1972), whereas the Q_{10} for heterotrophic microzooplankton is usually placed between 2 and 3 (Huntely and Lopez 1992), a factor that can also be assumed for bacteria as a compromise on the widely ranging estimates for bacterial temperature dependency (Li 1998). It is therefore assumed that heterotrophic plankton shows a stronger response to changes in ambient temperature than mainly light-controlled autotrophic phytoplankton, which might result in the de-coupling of former functional links and mismatch situations within the planktonic community, but also in changes of abundance and community composition. This in turn might influence the carbon pathways within the microbial food web and therefore the marine carbon cycle itself. An increasing bacterial production could, for example, lead to enhanced remineralisation of organic carbon and therefore a diminished export to the ocean depths. Similar alterations in the marine carbon flux could be induced by shifts within the bacterial community composition towards species which are better adapted to increasing ambient temperatures.

Rising temperatures due to ongoing climate change have been much discussed recently. According to the IPCC report 2001, north-central Europe will be most exposed to the effects of global warming during winter, with a predicted increase in sea surface temperatures of 5-10°C in this region for the ‘business-as-usual-scenario, which accounts for

a doubling of CO_2 emissions through out the current century (Houghton et al. 2001). Since the on-set of the algal spring bloom temporally coincides with this prognosticated increase in water temperature, information about the response of the complex interactions within microbial food web during the particular period of the early year is crucial for specifying the influence of rising temperatures on the marine carbon cycle.

The objective of this study was therefore to shed light on the response of the marine planktonic community to increasing ambient temperatures. Since we were not able to establish temperature controlled conditions in situ, we opted for analysing the development of a natural winter plankton community in an experimental system of several mesocosms, which enabled us to install a temperature gradient within the IPCC-predicted range of future rising temperatures. Taking cell counts by microscopy, the quantitative effect of rising water temperature on phyto- and bacterioplankton, virus like particles (VLPs), protozoa and metazoa during an artificial spring bloom was evaluated. Since bacterial cell counts alone are not necessarily sufficient to reliably determine the quantitative influence of temperature on bacterioplankton, actively respiring bacteria were assessed as well. Furthermore, we used molecular biological methods to analyse the bacterial and zooplanktonic community composition.

Materials and Methods

Experimental setting and sampling

During January 2006, eight indoor mesocosms were set up pairwise at four differently temperated climatic chambers for 26 days to simulate rising ambient temperatures. These four different treatments (Δ 0, Δ+2, Δ+4 and Δ+6°C) were determined by the initial temperature difference from the decadal mean from 1993-2002 in Kiel Bight. From the Kiel Fjord (IFM-GEOMAR pier, Kiel, Germany), water from 6 m depth was collected and evenly partitioned between the 1200 l-tanks synchronously, using a hose set. Sampled water was not filtrated previously to filling the mesocosms to ensure the collection of the entire planktonic winter community. Ammonium was added up to 21 µmol L^{-1} in order to meet the nutrient conditions of a similar experiment conducted the year before (Wohlers et al., unpublished results). All mesocosms were exposed to the same light regime of a day-night-cycle of 12:12 hours. During exposure, light increased for six hours from ~155 to ~388 µE m^{-2} s^{-1} and was reduced again down to ~155 µE m^{-2} s^{-1} during another six hours. Continuous mixing was provided by

one propeller in each tank. Daily sampling took place over the algal bloom, which began five days after incubation and lasted three weeks. Withdrawn water was not replaced in order to prevent a dilution of the initial water body as well as the adding of additional nutrients or organisms. Using teflon hoses for sampling, up to ten litres of water were siphoned directly into polycarbonate canisters. Samples for DNA- and CARD-FISH-filtration as well as counts of bacterial cells heterotrophic nanoflagellates and viruses were taken on a daily basis, whereas CTC-incubation was conducted every two days, as was sampling for phytoplankton. Three parallel samples for zooplankton counts were taken once a week with a mini-Apstein net (64µm mesh) and fixed with industrial methylated spirit.

Environmental parameters and inorganic nutrients

Temperature and salinity were measured daily and directly in the water column of the mesocosms using a Microprocessor conductivity meter LF 320 with a standard-conductivity cell (TetraCon 325; WTW, Weilheim, Germany). A pH meter with pH-electrode(SenTix 81; WTW, Weilheim, Germany) was used to assess the pH, also on a daily basis.

Unfiltered water samples were used for the measurement of ammonium as described by Holmes and colleagues (14). For the determination of nitrate, nitrite, phosphate and silicate (Hansen and Koroleff 1999), water was first filtrated through 0.65 cellulose acetate filters. All measurements were done on the day the sampling took place or after storage at -20°C until further progressing.

Enumeration of the planktonic community

After fixation of 2x100mL of sampled water with 0,5mL lugol solution, algal counts were performed using the inverted microscope method (Utermöhl 1958) for phytoplankton larger than 5µm. To attain 95% confidence limits of ± 20%, 100 individuals were counted for each taxonomic unit, which was not practical for rare species, however. For biomass calculations, the most abundant diatom and flagellate species were chosen. After approximation to the nearest geometric standard solid (Hillebrand et al. 1999), linear measurements were used to calculate algal cell volumes which were converted into carbon content (Menden-Deuer and Leassard 2000).

Total bacterial numbers were assessed after filtration of 1 mL of sampled water over 0.2 µm polycarbonate-filters. Cells were stained with DAPI (4´-6-diamidino-2-phenylindole) and

counted (1000x magnification) by epifluorescence microscopy (Axioskop, Zeiss, Germany). On average, between 600 and 850 not-attached (NA) bacterial cells were enumerated in a minimum of 20 and 15 view fields.

For the enumeration of heterotrophic nanoflagellates, aliquots of 15-20 ml were fixed with pre-filtered (0.2 µm pore-size) glutardialdehyde (EM grade, 1% final concentration) and filtered on black polycarbonate filters (0.8 µm pore-size), where cells were stained with DAPI (1 µg ml^{-1} final concentration). On those filters, several 'strips' of 5 to 15 mm were counted, using an epifluorescence microscope (Axioskop, Zeiss, Germany), with heterotrophic nanoflagellates being defined as flagellate-sized cells without visible chlorophyll which was assessed by its autofluorescence. On average, between 100 and 140 cells were counted per view field, a number which declined and increased depending on the stage of the algal bloom.

To assess the abundance of virus like particles (VLPs), 2 ml aliquots from 50 ml samples (fixed with pre-filtered (0.2 µm pore-size) glutardialdehyde, EM grade, 1% final concentration) were filtered over 0.2 µm polycarbonat filters to remove algal biomass and particles that might interfere with staining and visualizing VLPs. The thus pre-treated samples were finally filtered on Anodisc filters (0.02 µm pore-size, Whatman), where VLPs were stained with a mounting mixture of SYBR Green I and moviol (Lunau et al. 2005). VLPs were visualized and counted at 1000x magnification using an epifluorescence microscope (Axioskop, Zeiss, Germany). On average, between 70 and 110 VLPs were counted per view field.

Mesozooplankton was enumerated using a Leica MS5 binocular microscope. Samples were distinguished by stage of development into copepodites (C1 – C6, therefore including adult individuals) and copepod nauplii (N1 – N6) and corresponding eggs. Due to low initial mesozooplankton concentrations, we aimed at diminishing the population as little as possible. As a consequence, we were not able to keep the counting standards employed on phytoplankton, since the sample volumes within reasonable limits regarding the maintenance of the mesozooplankton community did not contain enough mesozooplankton individuals

Abundance of actively respiring bacterial cells

To assess the number of highly active cells, the fluorescent electron-transport system-specific reagent CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (Sherr et al. 1999) was applied to aliquots of 900 µL from each parallel and temperature at a final concentration of 4 mM.

Samples were incubated in sterile cryovials at the same temperature they've been taken at (Δ 0, Δ+2, Δ+4 and Δ+6°C) for two hours, and respiration was stopped with a mixture of paraformaldehyde (1% final concentration) and glutardialdehyde (0.05% final concentration). Samples were instantly frozen in liquid nitrogen and stored at –20°C until further processing. Enumeration of CTC-positive cells was done by flow cytometry, using a Becton&Dickinson FACScalibur (laser emission at 488 nm, constant flow rate (35 µl/min). Cells were detected in a plot of orange fluorescence (FL2) versus red fluorescence (FL3) (Bernard et al. 2000).

Filtration, isolation and PCR amplification of nucleic acids

Immediately after sampling, 250 to 300 mL of water were filtrated on two differently pore-sized polycarbonate filters (diameter 47mm, Osmonics, MN, USA). To remove large phyto- and mseozooplankton from the samples, the water was first filtrated over 3.0 µm and subsequently onto 0.2 µm polycarbonate-filters. The filters were shock-frozen in liquid nitrogen and stored at -20°C until further processing. Isolation of nucleic acids was performed by a pH-sensitive, slightly modified phenol-chloroform extraction to obtain DNA as well as RNA (Süß et al. 2006). For the phylogenetic documentation of the eukaryotic community, however, only DNA-samples were further processed by subsequent DGGE-PCR.

PCR for the bacterial as well as for the eukaryotic community was performed with a Bio-Rad my-cycler (Bio-Rad, Munich, Germany), using primer pairs specific for a 16S rRNA (358fGC, (Muyzer et al. 1993), and 907rM, (Muyzer and Smalla 1998)) and a 18S rRNA gene fragment (Euk1F and Euk516R-GC, (Diez et al. 2001)) of an approximate length of 500bp as is suitable for DGGE. For the amplication of bacterial nucleic acids, RNA was transcribed into cDNA by an RT-step, using iScript TM Select and the slightly modified included protocol (Bio-Rad, Munich, Germany; primer 1492r, (Lane 1991). Amplification of *Bacteria*-specific 16S rcDNA gene fragments was performed using a touchdown program with decreasing annealing temperatures from 65 to 55°C (2 cycles per step) and subsequent 16 standard cycles. 1 µl (1 to10 ng) of extracted DNA served as template. For amplification of the 18S rRNA gene fragment, 33 cycles of denaturation, annealing and extension (at 94°C for 2 min, 56°C for 45 sec and 72°C for 2 min followed an initial denaturation step of 2 min at 94°C slightly modified after the protocol described by Díez and colleagues (Díez et al. 2001).

For quantification, 4 µL of the amplification products, along with a quantitative ladder (1kB, Eppendorf), were analyzed by electrophoresis in 1,2% (w/v) agarose gels. The gels were stained with ethidium bromide and visualized under UV-light (Gel Doc XR, Bio-Rad, Munich Germany).

DGGE analysis and phylogenetic affiliation

The rather uniform DGGE banding patterns from a previously conducted similar experiment (AQUASHIFT spring 2005) did not suggest changes in the bacterial community composition at the transient temperatures Δ+2°C and Δ+4°C (Walther, unpublished results). It was therefore decided to constrain the genetic fingerprinting of the bacterial community to the two extreme treatments Δ+0°C and Δ+6°C.

DGGE of bacterial cDNA and eukaryotic DNA was performed with the Ingeny Phor-U system (Ingeny, Goes, Netherlands), using the slightly modified protocol described by Brinkhoff & Muyzer (Brinkhoff and Muyzer 1997). The denaturating gradient was adjusted to the specific assemblages (20-70% for the bacterial and 35-50% for the eukayryotic community). After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes, Inc.) and documented using the Gel Doc XR system (Bio-Rad, Munich, Germany). Prominent bands were excised and transferred into 50 mL of water (molecular grade, Eppendorf, Germany) in sterile Eppendorf caps using an ethanol-sterilized scalpel. Excised bands were incubated at 4°C overnight for resuspension of cDNA and finally stored at –20°C until further processing.

DGGE banding patterns of the bacterial and the eukaryotic communities were analyzed by performing Cluster analyses using the software Gel Compar II, version 2.5 (Applied Maths, Kortrijk, Belgium). Comparison of banding patterns was done curve based (Selje and Simon 2003) using Pearson correlation and UPGMA. Excised bands were sequenced by JenaGene (Jena, Germany) using the primer pair 358f and 907rM. For all sequences, at least 350 bp were analysed with the software SeqMan (DNASTAR). Phylogenetic affiliation was determined by using the BLAST function of the NCBI server (http://www.ncbi.nlm.nih.gov) to perform comparison with the data available in GenBank

Results

Environmental parameters and nutrient concentrations

Measurements of temperature exhibited values between 1.8 and 2.9 °C (Δ0°C treatment), 3.8 and 5.2 (Δ+2°C treatment), 5.7 and 6.7°C (Δ+4°C treatment) and 6.9 and 8.3°C (Δ+6°C treatment). The desired temperature gradient was established, but there was also a divergence between parallel tanks in all climatic chambers, being most pronounced in the warmest treatments. The development of the pH showed a slight increase from values between 7.8 and 8.0 in the initial stage of the experiment up to a pH of 9.2 at all temperatures over the course of the phyto-plankton bloom (Fig.1B). Salinity remained stable during the experiment at all four temperatures (Fig.1C), ranging between 18.1 and 18.7 during the sampling period and no notable differences between parallel treatments. Nitrate concentrations initially ranged between 20.8 and 21.4 µmol L-1 at all temperatures. A decrease began first in the Δ+6°C treatments at days 7 and 8 respectively, with NO_3^- being consumed to the detection limit within three and four days (Fig. 2 A).

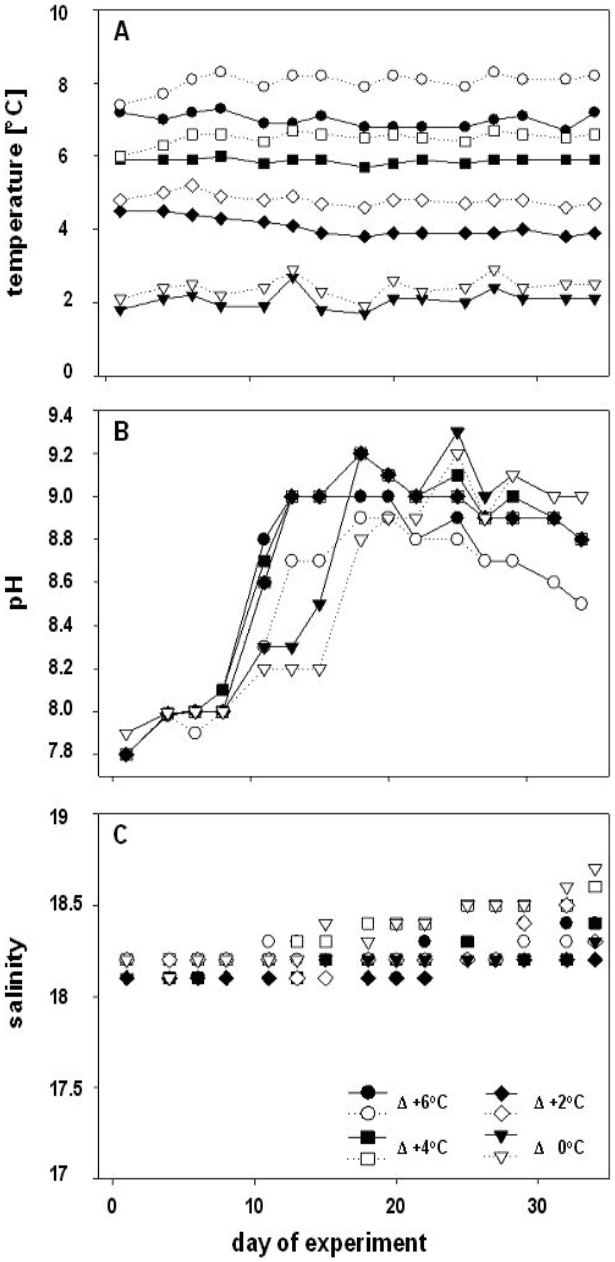


Fig.1: Development of environmental parameters measured during course of experiment: Temperature (A), pH (B) and salinity (C) for each temperature treatment; filled symbols representing parallel I, open symbols standing for parallel II.

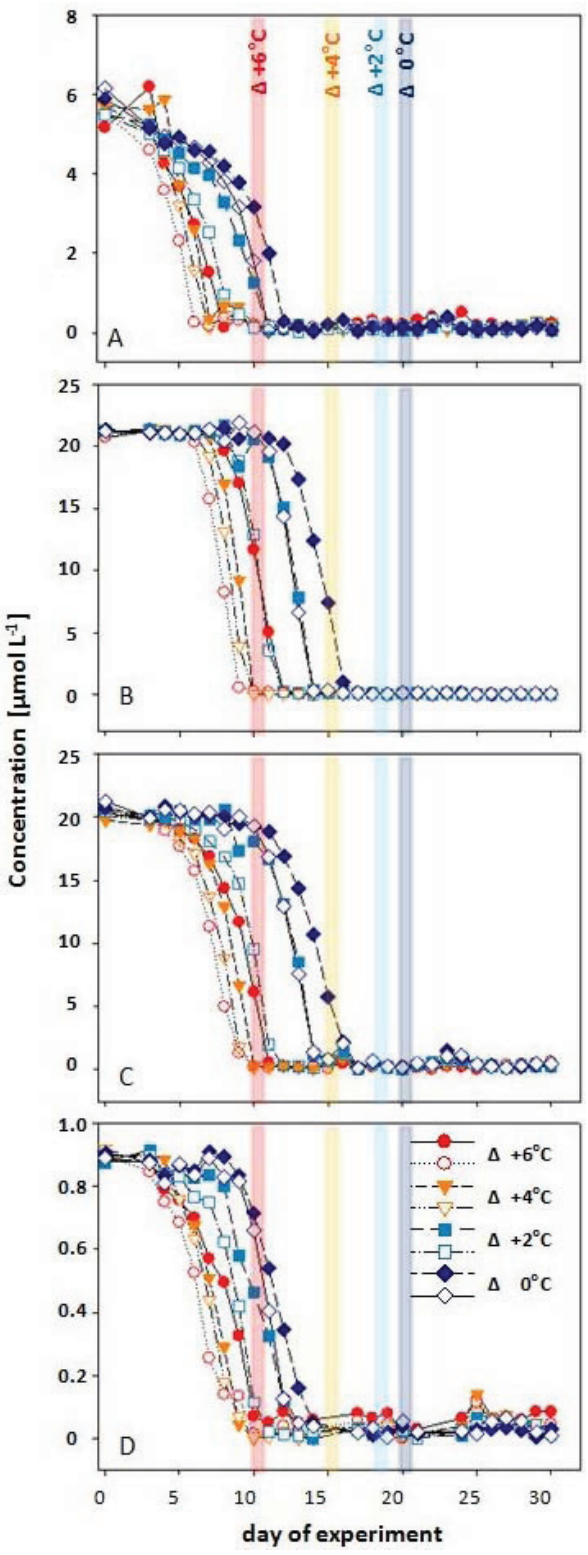


Fig.2: Dynamics of nutrient concentrations during course of experiment. Nitrate (A); ammonium (B), siliacte (C) and phosphate (D) concentrations for each temperature treatment; filled symbols represent parallel I, open symbols parallel II (data provided by Julia Wohlers); coloured bars mark peaks of algal blooms at different temperatures

The decline of NO_3^- started alter later in the colder treatments; at Δ0°C, values reached the detection limit at day 14 and 17, respectively. Ammonium concentration decreased from the first day of the experiment from initial values of 5.2 to 6.2 µmol L^{-1} to the detection limit within four and five days in the Δ+6°C and Δ+4°C treatment and 7 and 9 days in the two cold treatments (Fig.2 B). The consummation of silicate (initial values of 19.8 to 21.3 µmol L^{-1}) and PO_4^{3-} (initial values of 0.88 to 0.89 µmol L^{-1}) exhibited similar trends of a later set-in of declining values at colder temperatures to the detection limit (Fig. 2 C and D). During the late sampling period, PO_4^{3-} concentrations showed a slight increase up to 0.14 and 0.07 µmol L^{-1} in warm and cold treatments, respectively.

Phytoplankton dynamics

Analysis of algal biomass showed the development of pronounced algal blooms at all four temperature treatments (Fig.3 A-D). Highest concentrations of algal C were reached in the Δ+2°C treatment with 6.6 x10^4 µg C L^{-1} (parallel I) as well as at Δ0°C, where both parallels revealed phytoplankton maxima of 5.5 x10^4 µmol C L^{-1}. Generally, the occurrence of the phytoplankton bloom showed a delay with decreasing temperatures.

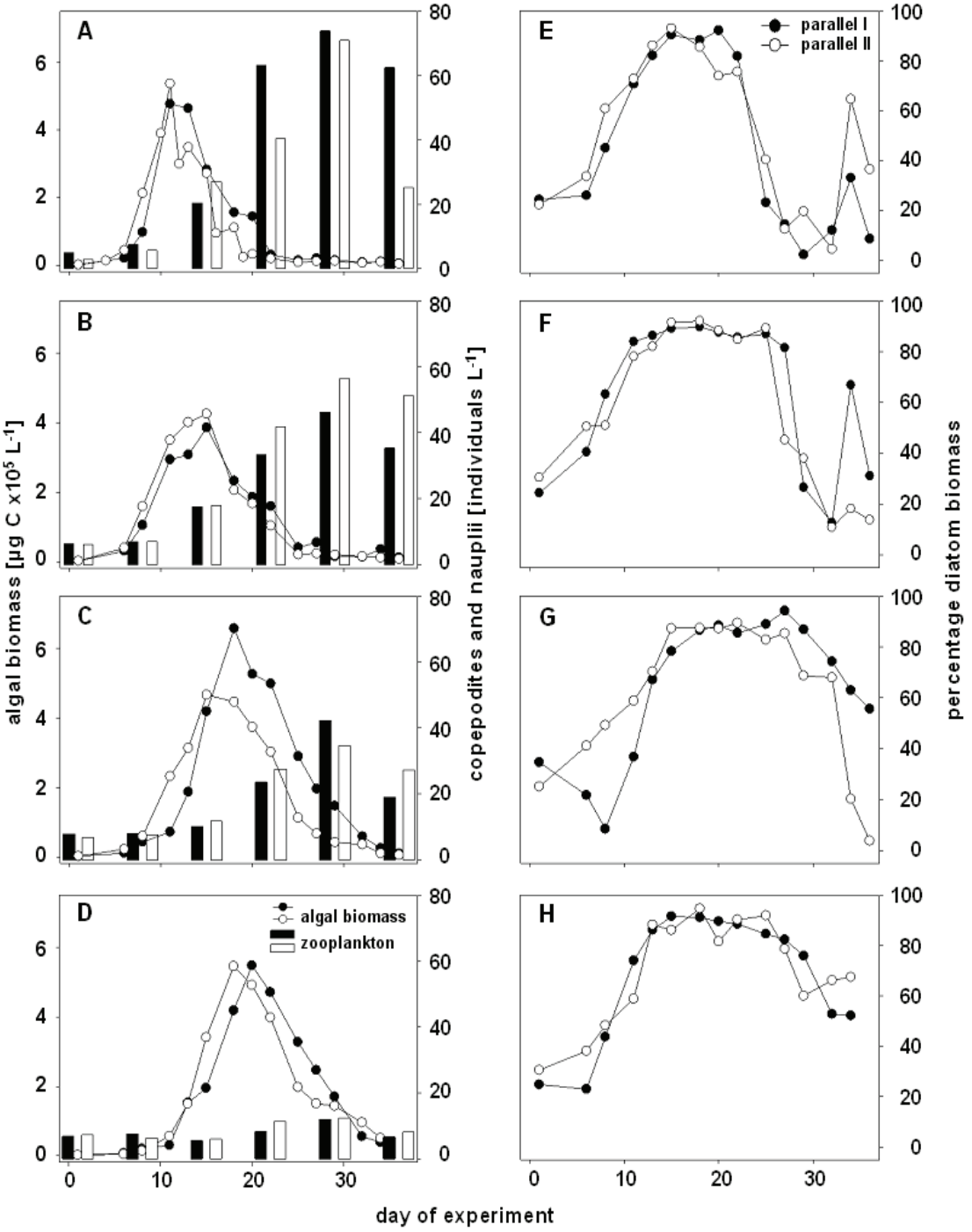


Fig.3: Dynamics of phyto- and metaozooplankton druing course of experiment. A-D show the parallel dvelopment of phytoplankton biomass (curves) and abundance of copepodites (C1 – C6) and nauplii (N1-N6) put together (bars) for the four temperature treatments Δ +6, Δ +4, Δ +2 and Δ 0°C. Dark symbols represent parallel I, open/white symbols stand for parallel II. E-H describe the share of diatom in total algal biomass, dark symbols representing parallel I, open symbols standing for parallel II (data on algal biomass provided by Ulrich Sommer).

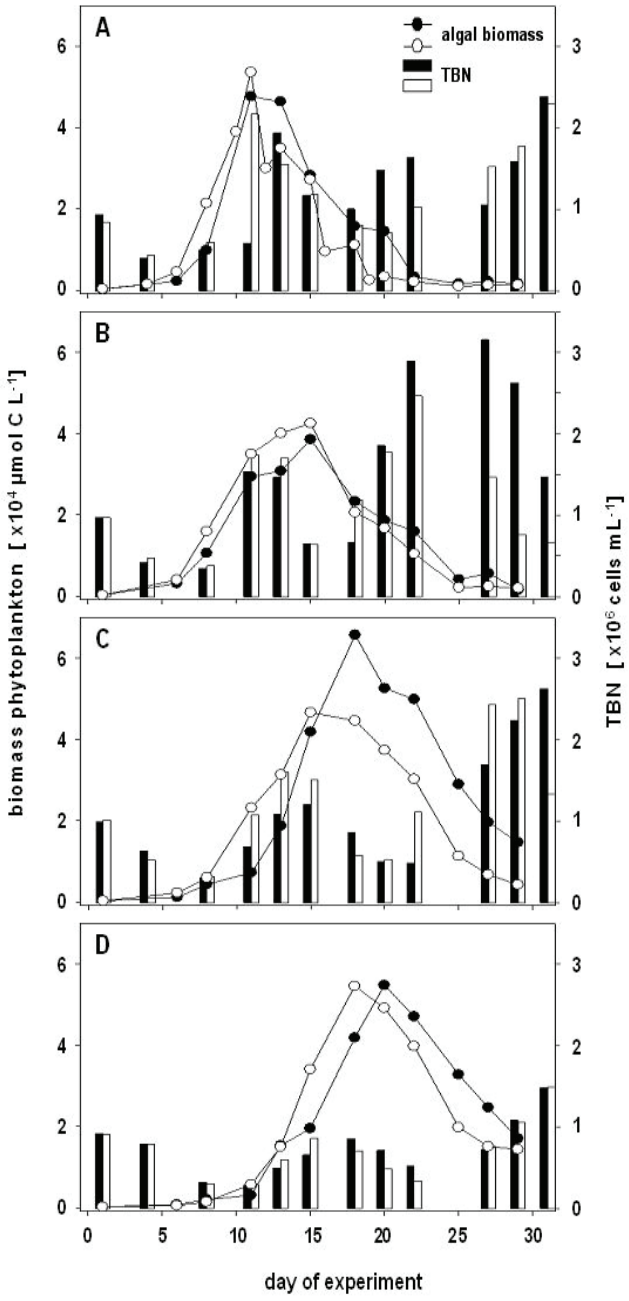


Fig.4: Development of phyto- and bacterioplankton during course of experiment. Plotted is algal biomass (curves); bars describe dynamics of total bacterial numbers (TBN) for the four temperature treatments Δ +6, Δ +4, Δ +2 and Δ 0°C (A,B,C,D). Filled symbols represent parallel I, active bacterial cells dropped by one order of magnitude between the first and the second peak (Fig.5 A-D; data on phytoplankton biomass provided by Uli Sommer)

Blooms took place earliest at Δ +6°C (day 11) and latest at Δ 0°C (day 18 to 20). In the two cold treatments (Δ+2°C and Δ 0°C), a time lag of several days was observed between the biomass maxima in parallel II and parallel I (Fig.3 A-D). Algal blooms were dominated by diatoms in all temperature treatments (Fig.3 A-D) with *Skeletonema costatum* being the most abundant species (data not shown). At all temperatures, the proportion of diatom biomass increased towards the peak of the bloom until it reached maxima of between 89 and 94% of the total phytoplankton biomass.

Dynamics of total bacterial numbers and actively respiring bacteria

Bacterial dynamics exhibited two peaks over the sampling period with the first occurring simultaneously with the phytoplankton maximum - though the bacterial peak at colder temperatures took place two days after the algal peak - and a second increase which occurred during the degradation phase of the algal bloom (Fig.4 A-D). Bacterial abundance was lowest in the Δ 0°C treatments where maximum cell counts of 1.78 and 1.63 x 10^6 mL^{-1} were reached. Highest TBN were observed during the second peak at Δ +4°C with 3.37 (parallel I) and 2.48 x 10^6 cells mL^{-1} (parallel II). There was a trend of slightly higher bacterial numbers with increasing temperatures during the first peak, whereas

the second bacterial peak did not show any link with ambient temperatures. Time lags between the phytoplankton peak and the second one of TBN were difficult to determine since, at least at the two cold temperature treatments, the bacterial numbers were probably still increasing after the sampling had been stopped.

The dynamics of CTC+ cells also revealed two peaks over the course of the algal bloom (Fig.5 A-D), following the TBN dynamics. Counts of CTC positive (CTC+) cells during their first peak were slightly higher at higher temperatures with maxima of 3.3 x 10^5 at Δ+6°C and 3.5 x 10^5 CTC+ cells mL^{-1} at Δ+4°C. In the Δ+2°C and Δ0°C treatments maximal counts were 3.1 x 10^5 and 2.4 x 10^5 CTC+ cells mL^{-1}, respectively. As already shown for the TBN, counts of highly active cells did not show a strictly temperature-related trend during the second peak. Highest values were assessed in the Δ+4°C treatment with a maximum of 5.1 x 10^5 CTC+ cells mL^{-1}; coldest tanks showed lowest counts with a peak at 3.6 x 10^5 CTC+ cells mL^{-1}. There was, however, a steeper decline of CTC+ cells at warmer temperatures (Δ+6°C and Δ+4°C), where the number of highly active bacterial cells dropped by one order of magnitude between the first and the second peak (Fig.5 A-D). This trend was not nearly as distinct in the Δ+2°C treatment and almost levelled out at coldest temperatures.

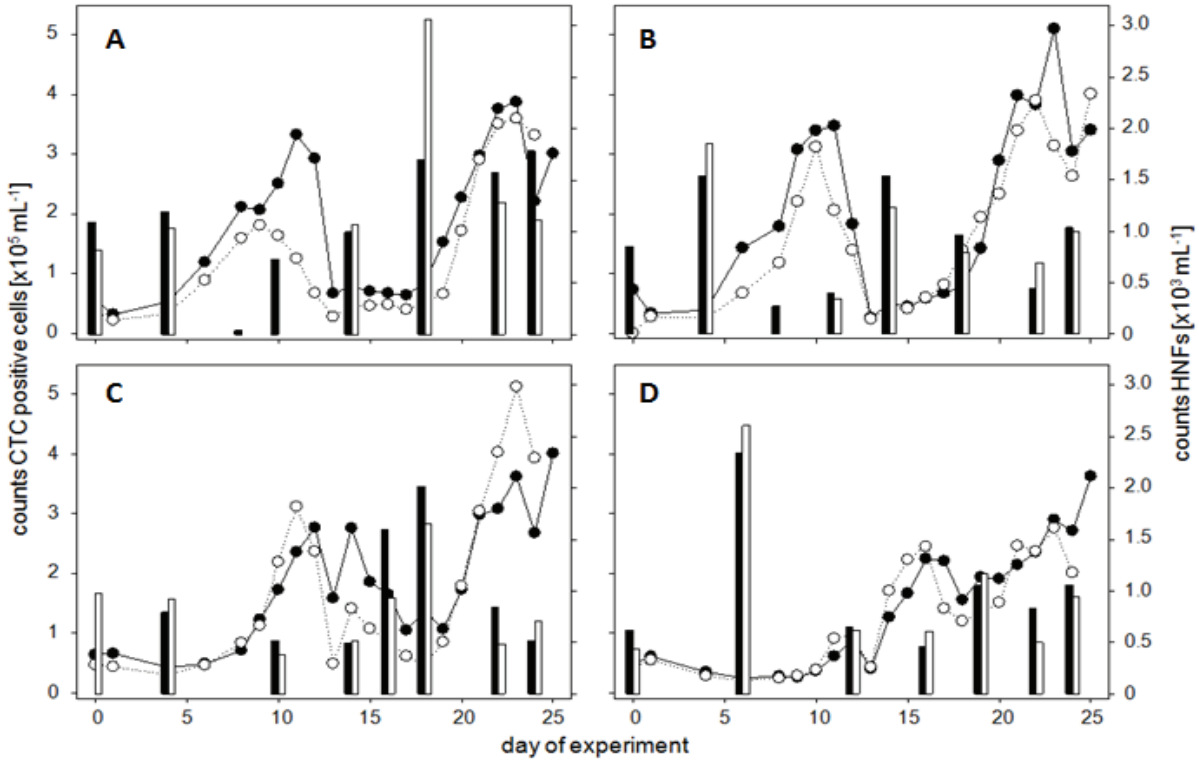


Fig. 5: Dynamics of highly active bacteria (CTC positive cells) and heterotrophic nanoflagellates (HNF) during course of experiment. Curves show the development of CTC positive cells, bars that of HNF for the four temperature treatments Δ +6, Δ +4, Δ +2 and Δ 0°C (A, B, C, D). Dark symbols represent parallel I, open/white symbols stand for parallel II.

The proportions of highly active cells of the total bacterial numbers mirrored that of the absolute values, also showing two peaks. The first co-occurred with the corresponding algal blooms at all temperatures with proportions ranging between 10.8 and 18.3 %. During the late stage of the bloom, proportions of highly active bacteria increased again, reaching percentages up to twice as high as during the first peak. With up to 38.6 % CTC^{+}-cells of TBN, the Δ 0°C-treatments exhibited the highest proportions of highly active cells (fig. 6).

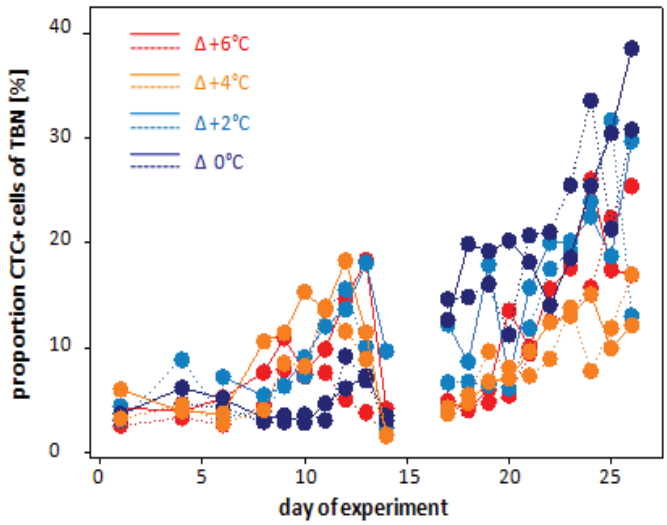


Fig 6: Percentages of highly active cells of total bacterial numbers for both parallels at all four temperatures (Δ +6, Δ +4, Δ +2 and Δ 0°C) over the course of the experiment. Lines represent parallel I, dashed lines parallel II.

Dynamics of heterotrophic nanoflagellatesand virus-like particles

Heterotrophic nanoflagellates (HNF) numbers reversely mirrored the development of CTC+ cells. Increasing abundance of highly active cells co-occurred with small HNF numbers, whereas growing HNF counts went along with a decline in CTC+ cells (Fig.5 A-D). Numbers of heterotrophic nanoflagellates during the initial phase of the sampling period were highest at Δ0°C with maximum counts of 2.34 and 2.61 x 10^{3} cells mL-1. HNF abundances after the breakdown of CTC-positive cells were not particularly linked with temperature and reached highest values in the Δ+6°C and Δ+2°C treatments with maximum counts of 1.7 and 3.1 and 2.0 and 1.7 x 103 cells mL^{-1}.

Counts of virus like particles (VLPs) assessed at three cardinal points during the first peak of actively respiring bacterial cells (during the exponential phase, at the peak, after the decline) remained fairly stable in the two warmer temperature treatments (Fig.7 A-D).

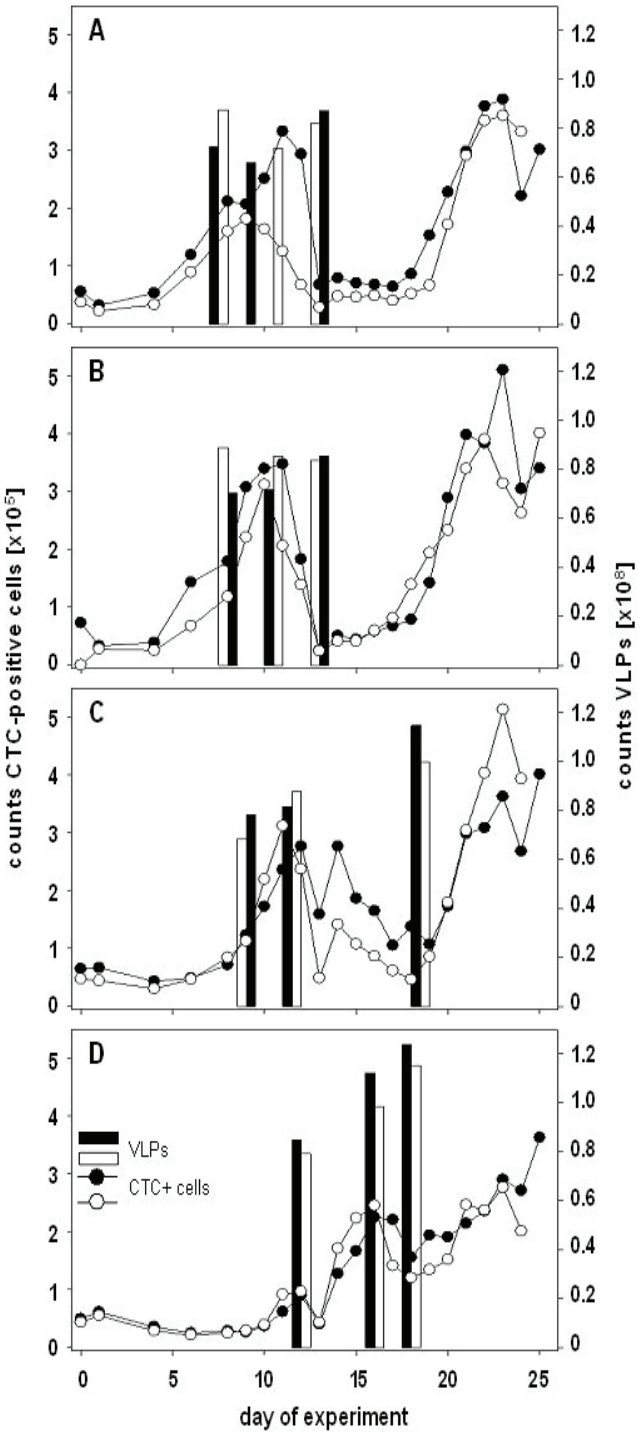


Fig.7: Development of highly active bacteria (CTC positive cells) and virus-like particles (VLP) during course of experiment for the four temperature treatments Δ +6, Δ +4, Δ +2 and Δ 0°C (A,B,C,D). Given are dynamics of CTC positive cells (curves) and VLP counts for beginning, peak and end of the first apex of highly active bacteria, with dark symbols representing parallel I, open/white symbols standing for parallel II.

In both parallels, the number of VLPs ranged between 0.72 and 0.87 x 10^8 cells mL^{-1} at Δ+6°C and 0.7 and 0.89 x 10^8 cells mL^{-1} at Δ+4°C. In contrast, there was a clear increase in VLPs over the three analysed samples at the two colder temperatures. At Δ+2°C, VLP counts increased from 0.72 and 0.64 to 1.15 and 0.99 x 10^8 cells mL^{-1}. In the coldest treatment, VLP abundance grew from 0,85 and 0.79 during the exponential phase of the first increase in CTC+ cells to1.24 and 1.15 x 10^8 cells mL^{-1}, respectively, after the decline of actively respiring cells.

Dynamics of mesozooplankton

To quantitatively determine mesozooplankton dynamics of nauplii and adult copepodes were analyzed together as one parameter. At all temperatures, counts revealed a community mainly consisting of *Oithona sp.* and *Pseudocalanus* with the latter dominating the assemblage. The *Pseudocalanus* community actually was a mixture of *Pseudocalanus sp.* and *Paracalanus sp.*, which are hardly distinguishable until adulthood. Yet, only very few adult *Paracalanus sp.* individuals were found at later stages of the experiment in all treatments.
Counting results revealed highest mesozooplankton abundances in warmest treatments with maximum counts of 70 and 76 individuals, respectively (Fig.3 A-D). With decreasing temperature, zooplankton numbers declined until at coldest temperatures, maximum counts of 11 and 12 in the respective parallels were reached (Fig.3 A-D).

In all tanks, mesozooplankton numbers increased during the decline of the phytoplankton bloom and showed maximum values at the end or even after (Δ+6°C) the algal degradation phase (Fig.3 A-D). This is in accordance with the notion that mesozooplankton mainly grazes on phytoplankton.

Microbial community structure

For qualitatively analysis of the bacterial and eukaryotic communities at crucial stages of the phytoplankton bloom, severalcrucial points of the overall algal development were chosen (bloom on-set, middle of the exponential phase, peak, middle and end of degradation phase). Samples from each parallel and temperature treatment taken at these points were examined by genetic nfingerprinting. Since the algal blooms lasted longer at cold temperatures, two points from the exponential and degradation phase were analysed instead of one in the warm treatments.

Genetic fingerprinting specific for *Bacteria* revealed 16 and 20 at Δ+6°C and 14 and 21 different bands at Δ 0°C (Fig. 8). There were several bands persisting during the entire course of the phytoplankton bloom, irrespective of temperature. The twelve excised and sequenced bands from the not-attached bacterial community revealed two chloroplast-derived ribotypes (AS06-12NA, AS06-19NA) and two bands that could be assigned to prymnesiophyceae (AS06-20NA, AS06-24NA), despite of the pre-filtration over 3.0 µm (Tab.1). The remaining bacterial-derived bands could be affiliated with the α-*Proteobacteria*, with three ribotypes (AS06-14NA/-23NA and AS06-18NA) assigned to the *Roseobacter* clade affiliated RCA-cluster (Selje et al. 2004), and the *Bacteroidetes* phylum (AS06-15NA;

AS06-16NA). Band AS06-22NA was closest related to the β-*proteobacteria,* with *Hydrogenophaga pseudoflava* being the closest cultivated relative (96%).

Excluding the algal-derived bands, the comparison of the banding patterns of the bacterial genetic fingerprint by cluster analysis showed two main clusters (Fig.9). The first solely included samples from parallel II from the Δ 0°C treatment, whereas the second consisted of samples from both extreme temperatures, indicating that the parallel I communities at Δ+6°C and Δ 0°C were more similar than the two cold bacterial assemblages among each other.

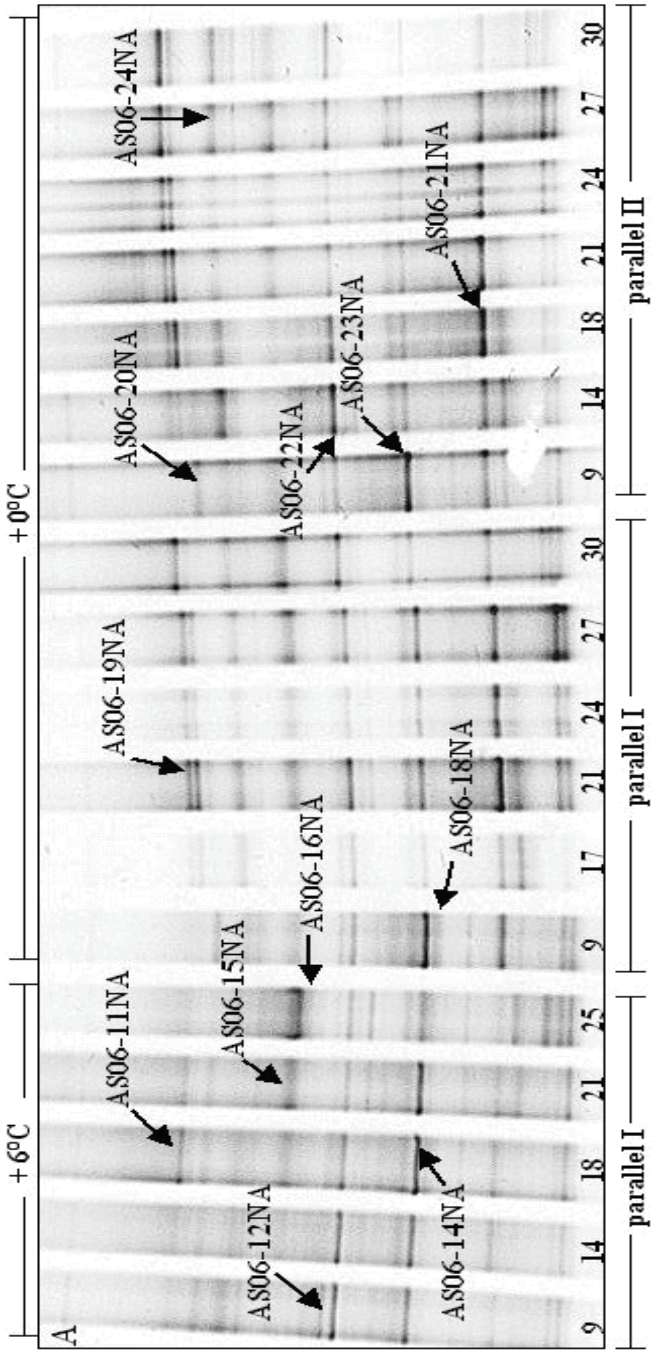


Fig. 8: Genetic fingerprint of the not-attached bacterial community at the two temperature extremes. Shown are 16S rRNA based c-DNA DGGE fingerprints done with the eubacterial primers 358f GC and 907rM at five selected cardinal points of the algal bloom, which are given as numbers over each lane (day of experiment). Arrows mark excised bands. Samples were taken for parallel I and II from the Δ 0°C and for parallel I from the Δ +6°C treatment.

Tab. 1: Phylogenetic affiliation, band identification (Band ID) of excised bands for the bacterial not-attached [NA] fraction at the extreme temperatures and their accession numbers. Further given are closest (cultivated) relative with accession number, similarity to them, their taxon and habitat.

Band ID	accession no.	closest cultivated relative (acc. no.)	similarity [%]	taxon	habitat/environmental features (closest relative)
AS06-11-NA	EU068014	*S.pseudocostatum* ; X82155	97%	eukaryota	marine, globally distributed
AS06-12-NA	EU068015	Guillardia theta ; AF041468	95%	eukaryota	marine
AS06-14-NA	EU068016	unc. *Rhodobacteraceae* bact. clone RCA-H28;DQ489286	99%	α-*Proteobact.*	marine, temperate and polar regions
AS06-15-NA	EU068017	*Aureimarina marisflavi*; EF108215	96%	*Bacteroidetes*	marine
AS06-16-NA	EU068018	*Winogradskyella thalassocola* ; AY771731	77%	*Bacteroidetes*	marine
AS06-18-NA	EU068019	unc. *Rhodobacteraceae* bact. clone RCA-11-5; AY165489	97%	α-*Proteobact.*	marine, temperate and polar
AS06-19-NA	EU068020	*Lauderia borealis* ; AJ536459	99%	eukaryota	marine, globally distributed
AS06-20-NA	EU068021	*Imantonia rotunda*; AY702162	86%	eukaryota	marine
AS06-21-NA	EU068022	*Hydrogenophaga pseudoflava* ; AF078770	96%	β-*Proteobact.*	fresh water
AS06-22-NA	EU068023	unc. *Rhodobacteraceae* bact. clone RCA-H28;DQ489286	99%	α-*Proteobact*	marine, temperate and polar
AS06-23-NA	EU068024	unc. *Rhodobacteraceae* bact. clone RCA-11-5; AY165489	97%	α-*Proteobact.*	marine, temperate and polar
AS06-24-NA	EU068025	*Imantonia rotunda*; AY702162	97%	eukaryota	marine

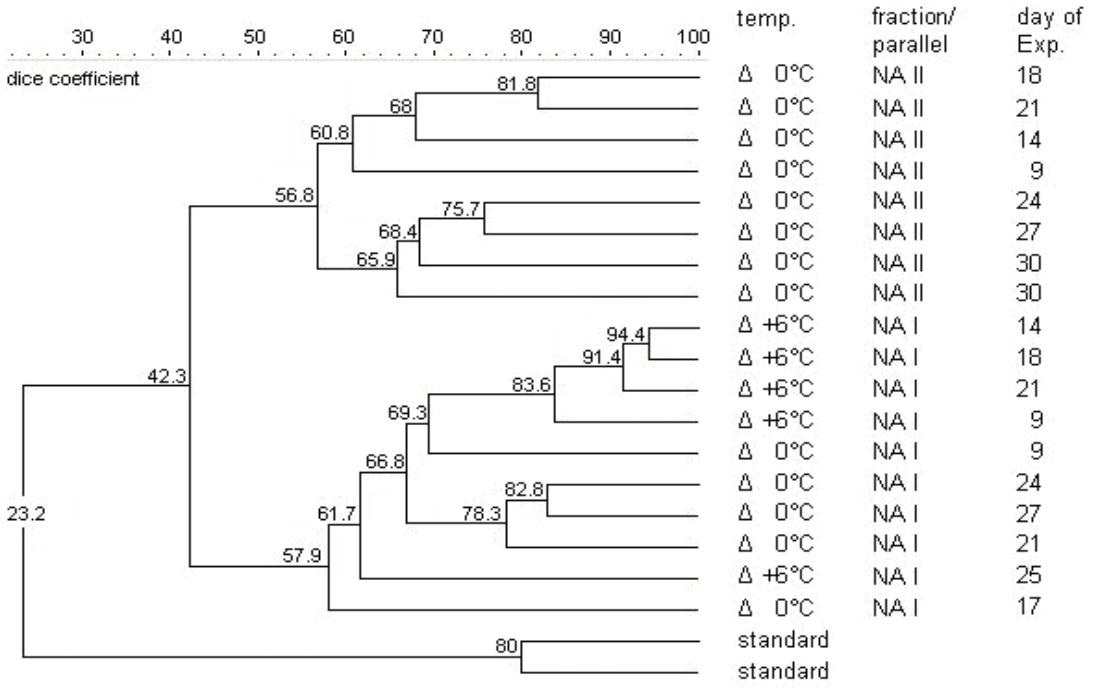


Fig.9: Cluster analysis of the genetic fingerprint shown in Fig.8. The dendogram shows similarities between the banding patterns for the two temperature extremes, with samples taken for parallel I and II from the Δ 0°C and for parallel I from the Δ +6°C treatment. Further given are day of experiment and similarity values within the dendogram.

For the eukaryotic communities, DGGE fingerprinting showed a rather low richness which apparently was not related to temperature (data not shown). Unlike with the bacterial assemblages, there were only very few bands that persisted over the course of the algal bloom and at all temperatures.

From the 14 bands excised from the eukaryotic fingerprint (data not shown), two were affiliated with the cryptophyceae *Rhodomonas* sp. (AS06-EUK3) and *Teleaulax* sp. (AS06_EUK7) and one was related to the dinoflagellate *Katodinium rotundatum* (AS06_EUK4). The ribotype AS06_EUK10 and AS06_EUK12 were affiliated with a marine gorgonial coral (*Junceella squamata*) and there were sequences related to gastrapoda (AS06_EUK18) as well as barnacles (AS06_EUK22). The remaining bands were all affiliated with different copepod species, even though one of those ribotypes was related to a freshwater species (AS06_EUK27).

Cluster analysis showed two main clusters, of which one included all samples from the Δ +6°C and most of the Δ +4°C treatments (Fig.10). All samples from the Δ 0°C grouped in the second cluster, along with one from the Δ +4°C mesocosms. The eukaryotic community in

the Δ +2°C tanks was evenly distributed over both clusters, forming sub clusters with samples both from the Δ +4°C as well as the Δ 0°C treatments.

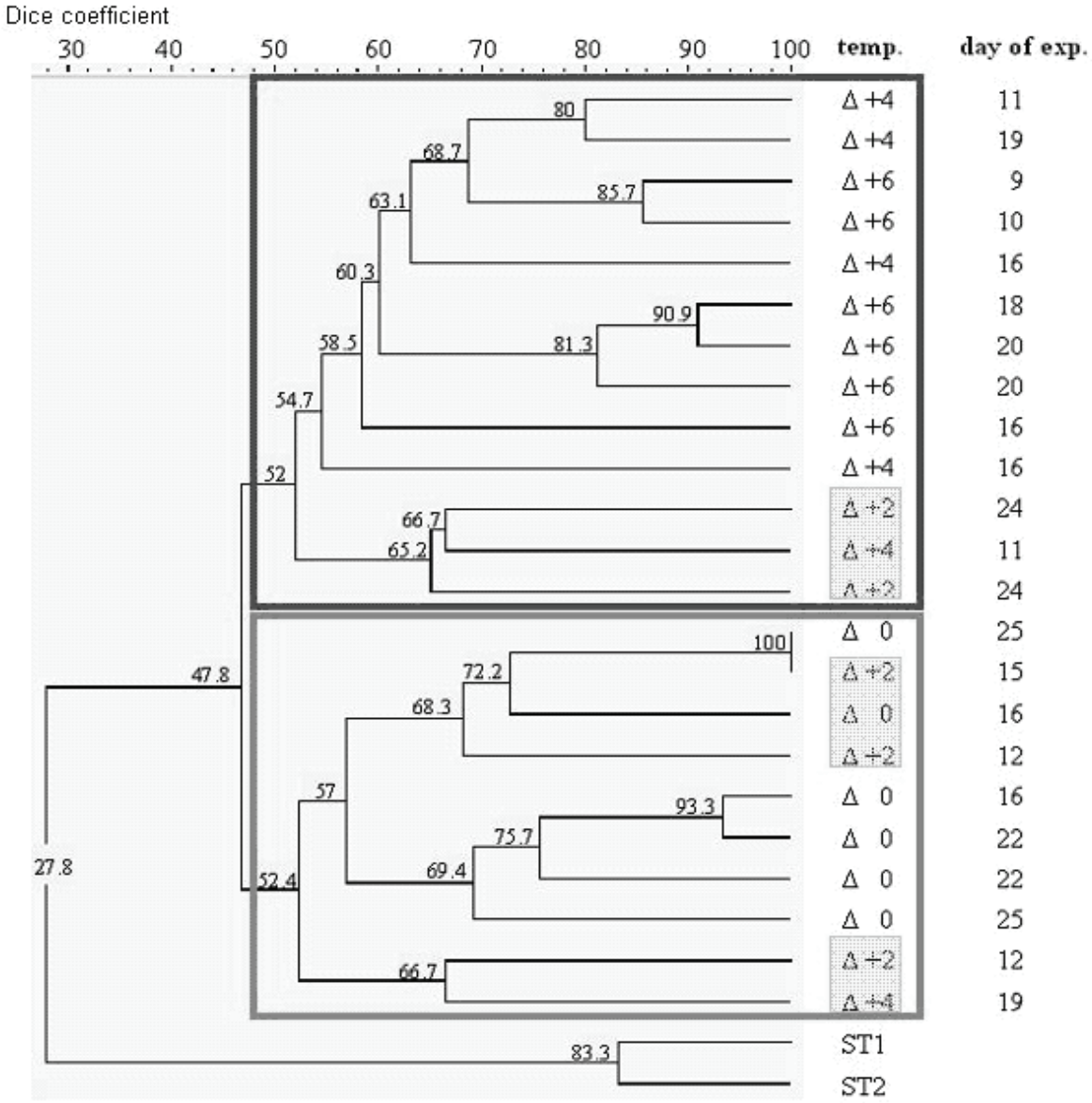


Fig. 10: Cluster analysis of the genetic fingerprint of the eukaryotic community. The dendogram shows similarities between the banding patterns for all temperature treatments, with samples taken at chosen cardinal points of the algal bloom, which are given as day of experiment. Numbers within the dendogram represent similarity values within the tree.

Discussion

In general, our experimental design has proven to be a reliable system to simulate the development and succession of the plankton community during an algal spring bloom along a temperature gradient. At all temperatures, depletion of nutrients was accompanied by distinct algal peaks; the subsequent development of bacterioplankton and HNFs, as well as that of metaozooplankton, followed the phytoplankton blooms. Though similar in general dynamics, there were quantitative differences in the planktonic development that seemed to be related to temperature. Algal biomass was highest in the coldest treatments, whereas counts of metazooplankton increased with rising temperatures. TBN were lowest at coldest temperatures, and the dynamics of highly active bacteria revealed a distinctly temperature-related pattern with a marked break-down at warmest treatments which was leveled out with decreasing temperatures. Within all planktonic communities, the quantitative development was delayed at coldest temperatures. General patterns like successions and over all dynamics therefore showed analogical trends in all treatments but varied in extent and timing according to temperature conditions.

The temperature gradient that was set up by the use of differently temperated climate chambers held over the duration of the experiment, except for one parallel of the warmest treatment which deviated towards a development closer to the Δ +4°C mesocosms. The developing algal bloom was dominated by diatoms and Skeletonema costatum, respectively. In the Kiel Bight, phytoplankton spring blooms have been shown to be of a similar composition: in an analysis of bacterial incorporation of organic substances in the Kiel Bight , Wolter (1982) found the phytoplankton community dominated by diatoms as well as the presence of *Skeletonema costatum* as one of the prevalent species. In a comparison of data from literature over the entire past century with recent analyses from the Kiel Bight, Wasmund and colleagues (2008) also observed *Skeletonema* one of the relevant algal species during spring blooms. In terms of phytoplankton biomass, a comparison between the results of the mesocosm experiment and a typical situation in the Kiel Bight is hardly feasible. Regarding the wide range of algal biomass Wasmund and colleagues (2008) found during the spring blooms in the Kiel Bight over three consecutive years (2001 – 2003), it is difficult to find representative data.

The bacterial community examined in this experiment resembled one expected in a brackish system. The excised and sequenced prokaryotic bands from the bacterial genetic fingerprint were dominated by members affiliated with the CFB (**C**ytophaga-**F**lexibacter-**B**acteroidetes) group but also those from subgroups from the *Proteobacteria*, with at least one representative of the β-*Proteobacteria* as a typical member of a freshwater community. These groups are common in brackish environments in general (Bouvier & del Giorgio 2002) and have also been found in studies conducted in estuarine waters in the Baltic Sea area (Kisand et al. 2002). The assumption that therefore the bacterial community that developed during this mesocosm experiment was no artificial assemblage is moreover substantiated by Riemann and colleagues (2008) who, by molecular as well as cultivation based analysis, found the bacterial community composition of the estuarine central Baltic Sea dominated by *Bacteroidetes*, but also detected typical fresh water species such as β-*Proteobacteria* We therefore assume that the experimental set up we used was sufficient regarding the objective to analyze the development of a planktonic community during an algal spring bloom under the influence of different ambient temperatures.

Temperature effect on single plankton compartments

Rising temperatures had a clear effect on phytoplankton development. The delay of several days regarding the timing of algal biomass peaks was markedly linked to temperature. This indicated that light conditions during the experiment were not limiting algal growth, which resulted in a temperature controlled phytoplankton development. Since diatoms dominated the blooms in all mesocosms and are known to form distinct blooms at colder ambient temperatures in general (Lomas and Glibert, 1999), it is not surprising that algal biomass was highest at the two cold treatments.

Heterotrophic bacterial development is known to be affected by temperature (Eiler et al. 2003), but also by nutrient concentrations (del Giorgio & Cole 1998), quantity and quality of organic substrate concentrations as well as viral lysis and grazing pressure (Weinbauer et al. 1998). The first peak in TBN which appeared at all temperatures was most likely triggered by substrate input derived from algal exudates (Baines and Pace 1991). Quantitatively, this released DOC should have been most pronounced at cold temperatures, where highest algal biomass was found, and lowest in the two warmer treatments which exhibited smaller concentrations of phytoplankton biomass. Still, highest TBN were found at high and lowest TBN at low temperature treatments, hinting at temperature having affected quantitative

bacterial development. Also, the general dynamics of highly active bacteria (Fig.5) appeared to be linked to the applied temperature gradient with the most prominent declines in CTC+ cells at the two warmer temperatures and a comparatively consistent development in the coldest treatment. These different dynamics were not mirrored in the rRNA-based genetic fingerprints, though, where the cluster analysis of the two extreme treatments (Δ +6 and Δ 0°C) showed very similar respective bacterial NA communities. Any direct or indirect temperature influence was apparently a solely quantitative one. Since neither growth rates nor grazing experiments have been conducted, temperature dependent protist or ciliate grazing cannot be excluded as a potential reason for the development of TBN or the dynamics of highly active bacteria. Though the counts of HNFs do not hint at it (see below), it is still possible that low temperatures resulted in diminished grazing acticity, with HNFs and/or Ciliates mediating a therefore indirect temperature effect to the food web level below them. Without reliable grazing data, this question cannot be conclusively answered.

The development of potential bacterial grazers and lysis apparently responded differently to the temperature gradient. Heterotrophic nanoflagellates and VLPs did not show any constant temperature related trends, with the HNF community revealing an only very slight decline at coldest temperatures. The metazooplankton community apparently was affected quantitatively by increasing warmth of the environment. The eukaryotic genetic fingerprint which was predominated by metazooplankton (presumably, the proportion of HNF nucleic acids in the samples was not high enough to have been sufficiently amplified by PCR, therefore resulting in a lack of protist signals in the eukaryotic fingerprints) furthermore suggested that the composition of the assemblages from the two extreme temperatures differed from each other, whereas those from the Δ+4 and Δ+2°C treatments seemed to represent a kind of intermediate community, clustering with both coldest and warmest samples. Temperature therefore apparently affected metazooplankton on both a quantitative as well as qualitative level.

The impact of temperature on the planktonic community

The different planktonic communities we analyzed are tightly linked among each other by grazer-prey and virus-host relations (Weinbauer and Höfle 1998). These couplings are known to have an impact on abundance as well as community structure, especially of the prey/host assemblage (Matz and Jürgens 2003). Environmental aspects affecting the grazer/virus communities can therefore indirectly also have an impact on the prey/host organisms. To

reliably determine the influence of rising temperatures within the planktonic food web, it is therefore necessary to also take those affects into account which might be indirectly mediated by other organisms or factors.

The quantitative analyses of the planktonic communities in the presented experiment suggested similar interactions of grazer/prey organisms as described above. The most pronounced temperature-related changes that were found within the plankton assemblage were the difference in the dynamics of highly active bacteria between the two temperature extremes. The pronounced break down of CTC+ cells after the first peak as a general trend for this parameter clearly decreased with falling temperatures, but appeared to be rather a result of grazing or viral lysis (or lack thereof) than of an direct impact of temperature at first sight. The dynamics of heterotrophic nanoflagellates did not support this notion, though (Fig.5). HNF numbers increased after the decline of CTC+ cells in all temperature treatments, but highest counts have been taken in the Δ+2°C where the break-down of highly active bacteria already begun to level out, whereas the differences in HNF numbers between the two warm and the coldest tank were not pronounced enough to allocate the drastic differences in CTC+ cell dynamics to HNF grazing. These results indicate that, as expected, nanoflagellates actually did prey on bacteria, but were not mainly responsible for the distinctly different CTC+ dynamics along the temperature gradient, even more so since there were no pronounced temperature-related differences in HNF numbers themselves. Still, HNF counts from day six in the coldest treatment, which were markedly higher than at the other temperatures, might explain why CTC+ counts were clearly lower during the first peak in the coldest mesocosms compared to the others. However, since neither HNF species composition nor grazing rates are known for this experiment, this interpretation of the interaction between bacteria and HNF is based solely on the foundation of quantitative trends over the algal bloom and can therefore only be a partial one.

Viral lysis is a further factor for cell death among the bacterial community. Several studies have already shown that bacteriophages play a decisive role in quantitative as well as qualitative control of the bacterial community (Weinbauer and Höfle 1998, Winter et al. 2004), which might be as important as that of HNF grazing (Hahn and Höfle, 2001). There have also been studies on the effect of temperature on viral lysis of bacteria, which suggest temperature related changes in burst sizes and virus decay (Noble & Fuhrman 1997). Our results, however, seem not to be in accordance with that, though our analysis of VLPs might not have been extensive enough to make reliable deductions. The VLP counts that were taken

before, during and after the first peak of highly active bacteria did not indicate that viral lysis had a share in the break-down of CTC+ cells in the warmer tanks (Fig.6 A, B), even though VLP counts increased slightly in the Δ+2 and Δ 0°C treatments. Since it is assumed that the lysis of infected cells and the release of virus-like particles takes place in a more or less synchronous way (Bratbak et al. 1996), viral lysis as the reason for the break-down of highly active bacteria at colder temperature would imply a more pronounced increase in virus-like particle numbers.

It is well established that metazooplankton prey on phytoplankton. Though grazing by proto- and metazooplankton apparently not always controls the *in-situ* dynamics of algal biomass (Lignell et al. 1993), the phytoplankton development within the enclosed conditions of the presented study have been clearly affected by this grazing activity. The diminishing algal peak was mirrored by an increase in metazooplankton numbers (Fig.3 A-D); furthermore, highest phytoplankton biomass went along with low counts of metazoa in the two cold treatments, whereas highest numbers of metazooplankton resulted in a reduced algal development at Δ+4 and Δ+6°C. Aside from their impact on phytoplankton, it has been shown that metazoan grazing can also control bacterioplankton in freshwater environments (Thouvenot et al. 1999); further studies imply that at least several species of nauplii (NP), whose abundance clearly increased with growing temperatures in our experiment (Fig.3 A-D), are capable of bacterivory (Rolff et al. 1995). In the Δ+6 and Δ+4°C, the NP numbers showed their first clear increase around day 15, which coincides with the break-down of CTC+ cells at these temperatures. The only slight increase NP at day 15, coupled with over all lower numbers in the Δ +4°C treatment, is mirrored by a clearly reduced decrease of the first peak of CTC+ cells at these temperatures; in the coldest treatment, the rather weak increase in NP counts goes together with a diminished break-down of highly active bacteria. These results suggest that nauplii had a grazing impact on the bacterial community, yet it is more likely that ciliates, which were not counted for this experiment, were the major grazers on the highly active bacteria. If grazing was the actual reason for the break-down of CTC+ cells in the warmer tanks, the subsequent recovery of the highly active bacterial community (Fig.5) hints at the development of grazing resistance, probably by the reduction of cell size which might prevent retention in the filtering (Langenheder and Jürgens 2001). Since our genetic fingerprints show a rather conform visual picture over the course of the experiment (Fig.8), it is unlikely that new bacterial species better adapted to withstand grazing pressure by nauplii or HNFs took over the community. Cluster analysis also did not indicate a change in bacterial community composition with the progress of the algal bloom (Fig.9).

The development and succession of a plankton community during an algal spring bloom along a temperature gradient was successfully simulated. The obtained results suggest that at least for phytoplankton, the well-established grazer-prey interactions between algae and metazooplankton also took place during this experiment, probably mediating temperature impacts affecting the grazer on to the phytoplankton community. The marked seemingly temperature-related dynamics of highly active bacteria that were found remain unexplained, since the HNF-counts do not offer clarification, and ciliates as the second potential bacterial grazers have not been assessed in this study. A direct temperature effect on the highly active bacterial community can also not be categorically excluded.

In general, planktonic dynamics as shown here always represent an interplay of production on the one and mortality on the other hand. In order to find out whether and/or how temperature affects these dynamics, it is important to consider how the two afore-mentioned aspects constituting a quantitative community development answer to a temperature gradient. The factors responsible for cell death have been shown and discussed for this experiment. For phytoplankton, the interaction between algae and metazooplankton indicated that grazing was one reason for the break down of the algal blooms at all temperatures – albeit not related to temperature. Regarding bacterioplankton, and solely based on cell counts, a temperature dependency could neither be shown for potential HNF grazing nor for viral lysis. Bacterial cell death therefore seemed not to have been temperature-linked (it has to be mentioned again that specific grazing experiments have not been conducted and that ciliates have not been analysed at all in this study).

Taking a look at production as the second factor controlling community dynamics might shed more light on the apparently temperature-related dynamics of highly active bacteria. A more detailed investigation focused on bacterial production, as well as community composition under the influence of rising temperatures, including the particle-attached bacterial assemblage as well, will be necessary to get a more detailed impression on the effect of rising ambient temperature on the bacterial assemblage.

II. Temperature effects on the bacterial community composition during an algal spring bloom: a mesocosm study

Abstract

To examine whether rising ambient temperatures affect the phytoplankton-bacteria coupling and the bacterial community composition (BCC) during early spring bloom conditions, indoor mesocosm experiments were performed during which natural winter plankton communities from the Baltic Sea were incubated at two different temperatures (Δ 0°C and Δ+6°C from the in situ ten-year temperature average in the Kiel Fjord of 2°C). In this study, the composition of the not-attached (NA) as well as the particle-attached (PA) bacterial assemblage was studied during the course of an induced phytoplankton spring bloom by using the PCR-based genetic fingerprint method denaturing gradient gel electrophoresis (DGGE) as well as the catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) method. In the genetic fingerprints of 16S rRNA gene fragments, shifts that were linked to ambient temperatures were detected only within the particle-attached fraction, for which comparison of banding patterns revealed temperature-related clusters. Within the PA fraction, one ribotype solely present at cold temperatures was found. Sequence analysis revealed this band being affiliated to the β-*Proteobacteria*. On a group-specific level, our CARD-FISH counts implied that the dynamics of *Bacteroidetes* of both the NA and PA fraction were affected by temperature over the algal bloom, as well as the NA α-*Proteobacteria*. We therefore conclude from our analyses on the species- as well as the group-specific level that a temperature difference of 6 °C during an induced phytoplankton spring bloom resulted in shifts within the bacterial community composition. Since this effect was more pronounced in the PA fraction and also the share of PA bacterial production, measured by thymidine incorporation, increased up to 40% in cold and 28% in warm treatments over the progressing bloom, our study not only emphasises the relevance of the PA bacterial community during a phytoplankton spring bloom, but also reveales the relevance of the effect rising water temperatures have on the particle-attached bacterial assemblage.

Introduction

The interplay of phyto- and bacterioplankton in the functional coupling between primary production and bacterial degradation of algal derived organic carbon (van Es & Meyer-Reil 1982) is a fundamental facet of the marine carbon cycle. Degradation of algal-derived DOM and POM as well as bacterial production and respiration have great influence on the extent of 1) recycled carbon within the microbial loop (Azam et al. 1983), 2) refractory carbon being transported to the ocean depth and 3) CO_2 released to the atmosphere (Azam 1993). As with microbes in all ecosystems, heterotrophic bacterial activity within the microbial loop is affected by temperature, which might have considerable impact on the three aspects of the marine carbon cycle mentioned above. Yet, the role of temperature in the coupling of phyto- and bacterioplankton at low water temperatures is not clear, though its impact may be of high significance during a phytoplankton spring bloom in temperate zones. There is evidence that during a spring bloom event in cold waters, temperature might be one of the factors potentially suppressing bacterial activity (Pomeroy & Wiebe 2001), resulting in a decoupling of phyto- and bacterioplankton which in turn leads to a considerable proportion of algal derived carbon not being recycled (Pomeroy & Deibel 1986). In contrast to that, Morán and co-workers (Morán et al. 2001) documented a considerable coupling between algal production and bacterial consumption of DOC in Antarctic offshore waters, which was dependent in strength on the amount of coastal DOC input (Morán et al. 2002). In another study, Yager and Co-workers (Yager et al. 2001) also found a definite response in bacterial activity and abundance to an early algal spring bloom at subzero temperatures.

Irrespective of the effect of cold waters on the algal-bacterial coupling and its strength, global warming is predicted to increase especially North-Central European temperature particularly during winter and spring (Houghton et al. 2001). Therefore, it is likely to affect the interplay of phyto- and bacterioplankton during early algal spring blooms. Due to different Q_{10} factors, which commonly range between 2 and 3 for heterotrophic bacteria (Pomeroy & Wiebe 2001) and 1 and 2 for autotrophic phytoplankton in cold waters (Tilzer & Dubinsky 1986), rising temperatures presumably will result in a proportionally more enhanced bacterial activity than phytoplankton primary production, thereby leading to increased re-mineralization of phytoplankton-derived carbon within an intensified microbial loop, and as a consequence to a reduction of the amount of carbon exported to the ocean depth (Kirchman et al. 1995, Legendre & Lefevre 1995).

Due to the relevance of the microbial loop for the marine carbon cycle as described above, it is crucial to know whether and how changes in temperature might affect the performance of the bacterial community in terms of bacterial activity and community composition (BCC). The increase of heterotrophic bacterial activity with rising temperatures has been well studied. Several authors have been able to show that bacterial cell-specific production and respiration are highly temperature dependent (Felip et al. 1996, Pomeroy & Wiebe 2001, Kirchman et al. 2005, Lopez-Urrutia et al. 2006). There is only little and partly contradicting information about the influence of temperature on the bacterial community composition, though. Nedwell (1999) concluded that temperature affects bacterial substrate affinity, showing that the available pools of substrates actively taken up by bacteria are effectively limited at lower temperatures due to inhibited transport proteins in the cell membrane. Decreased substrate affinity at low water temperatures may therefore select for species with higher affinities for a given substrate than others, whereas the species that were outcompeted in the cold may grow abundant again with increasing temperatures (Nedwell 1999). Investigating community structure and growth of the bacterial community in the Southern Ocean, Simon and colleagues (1999) found different bacterial communities along a gradient of decreasing optimum temperature for bacterial growth. Some of the studies on aquatic bacterial community composition with regard to seasonal temperature changes showed significant influence of several bottom-up factors, including temperature (Muylaert et al. 2002, Yannarell & Triplett 2004), while others did not significantly link shifts in bacterial community structure with temperature (Zwisler et al. 2003, Henriques et al. 2006) or found rather stable communities over the year (Schauer et al. 2003). However, none of these studies concentrated on the influence of temperature alone, but included other environmental factors that might be subject to seasonal fluctuations as well. Therefore the particular effect of rising ambient temperatures on the composition and diversity of aquatic bacterial assemblages is still unknown.

To approach this question, an indoor mesoscosm experiment was set up, designed to simulate the dynamics of the planktonic community during a spring bloom event at two different temperatures. The purpose of this study was to determine whether an increase of 6°C from *in situ* water temperatures (+2°C) would affect the bacterial community composition during an algal spring bloom. By applying the molecular techniques CARD-FISH, DGGE-fingerprinting and sequencing of excised bands, we were looking for potential shifts within the structure of the bacterial assemblages as well as identifying abundant bacterial representatives at different temperatures on the group and the phylogenetic level.

Materials and methods

Experimental design, sampling and abiotic parameters

Eight indoor mesocsoms were set up pairwise in four differently tempered climatic chambers to allow parallel sampling. In order to simulate an increase in ambient temperatures, the chambers were set at a gradient, ranging from *in situ* temperature at approximately 2°C (calculated from the mean water temperatures of the Kiel Fjord at the beginning of February over ten years, termed Δ 0°C in the following) up to 6 °C above *in situ* temperature (Δ +6°C), increasing in steps of 2°C. From the Kiel Fjord (IfM-GEOMAR pier, Kiel, Germany), water from 6 m depth was collected and equally divided between the 1200 L tanks synchronously by using a hose set. To ensure collection of the entire plankton community, the water was not prefiltered. Ammonium was added up to 21 µmol L^{-1} in order to meet the nutrient conditions of a similar experiment conducted the year before (Wohlers et al., unpublished results). All mesocosms were exposed to the same light regime of a day-night-cycle of 12:12 hours. Light increased for six hours from ~155 to ~388 µE m^{-2} s^{-1} and was reduced again down to ~155 µE m^{-2} s^{-1} during another six hours. Continuous, gentle mixing was provided by one propeller in each tank. Samples were taken daily during the algal bloom, starting on day 5 until day 31 of the experiment. Withdrawn water was not replaced in order to prevent dilution effects on the initial water body and unwanted addition of nutrients or organisms. Using teflon hoses, up to ten litres of water were siphoned directly into polycarbonate canisters from which samples for DNA, CARD-FISH and bacterial cell counts were taken. Since the results from a similar previous experiment indicated stable bacterial communities at the temperature levels Δ+2°C and Δ+4°C (Walther, unpublished results), we decided to analyse here only the extreme treatments with temperatures of Δ+0°C and Δ+6°C.

Temperature and pH were measured using the Microprocessor conductivity meter LF 320 with a standard-conductivity cell TetraCon 325 and a pH meter with pH-electrode SenTix 81, respectively (both WTW Weilheim, Germany).

Chlorophyll *a* concentration

For the analysis of chlorophyll *a* (chl *a*), 50 to 500 mL of sampled water was gently (<200 mbar) filtered through GF/F filters which were stored at -20°C until analysis. For the analysis, pigments were extracted in 90% acetone and measured fluorometrically on a 10-AU fluorometer (Turner Designs, California) according to (Welschmeyer 1994).

Enumeration of bacteria and phytoplankton

From each water sample, 100 mL were fixed with formaldehyde (2% vol/vol final concentration) and stored at 4°C until further processing for enumeration of bacteria. Concentrations of total bacterial numbers (TBN) and particle-attached bacteria were assessed after filtration of 6 mL of pretreated water over black 0.2 µm polycarbonate filters. Cells were stained with DAPI (4′-6-diamidino-2-phenylindole) and counted under an epifluorescence microscope (Axioskop plus, Zeiss, Germany) at 1000x magnification. In a minimum of 20 and 15 view fields for NA and PA bacteria, respectively, between 600 and 850 bacterial cells were enumerated on average, a guideline that could not always been met for PA cells at early stages of the bloom when the occurrence of particles was very low. Algal cells were counted using inverse microscopy (Utermöhl 1958). To attain 95% confidence limits of ± 20%, 100 individuals were counted for each taxonomic unit, which was not practical for rare species, however.

Bacterial production

Incorporation of ^{3}H-methyl-thymidine for the determination of bacterial secondary production was done slightly modified after Fuhrman & Azam (Fuhrman & Azam 1982). For each sample, three replicates and one blank (treated with 1% v/v formaldehyde) of 10mL of water were each incubated with 50µL of a 1µCi/10µL ^{3}H-methyl-thymidine solution (specific activity: 63 µCi/nmol), resulting in a final and saturating concentration of 8 nmol L^{-1}. All samples were incubated in the respective climate chambers at in situ temperature in the dark for 1.5-3 hours and the incubation was terminated by the addition of formaldehyde (1% v/v). From each sample, 5mL were filtered through 3µm and 0.2µm polycarbonate filters, respectively. This was assumed to correspond to total bacterial production (0.2µm filter) and particle-associated bacterial production (3µm filter). The filters were rinsed with ice cold 5% TCA (trichloro-acetic acid) solution before being radio-assayed in 4mL of scintillation cocktail (Lumagel Plus) using a Packard Tricarb scintillation counter.

Filtration, isolation and PCR amplification of nucleic acids

Immediately after sampling, 500 to 600 ml water were filtered subsequently through two differently pore-sized polycarbonate filters (3.0 and 0.2 µm pore size, diameter 47mm) in order to distinguish between particle-attached and not-attached bacterial assemblages. The

filters were shock-frozen in liquid nitrogen and stored at -20°C until further processing. Isolation of nucleic acids was performed by a pH-sensitive, slightly modified phenol-chloroform extraction to obtain DNA as well as RNA (Süß et al. 2006). However, only RNA samples were processed further by first being transcribed into cDNA by an RT-step, using iScript TM Select and the slightly modified included protocol (Bio-Rad, Munich, Germany ; primer 1492r, (Lane 1991), and subsequently used as template for DGGE-PCR.

PCR was performed with a Bio-Rad my-cycler (Bio-Rad, Munich, Germany), using primer pairs specific for *Bacteria* 358fGC (Muyzer et al. 1993), and 907rM (Muyzer & Smalla 1998). Amplification of *Bacteria*-specific 16S rcDNA gene fragments was performed using a touchdown program with decreasing annealing temperatures from 65 to 55°C (2 cycles per step) and subsequent 16 standard cycles. One µl (1 to10 ng) of extracted DNA served as template. For quantification, 4 µl of the PCR products, along with a quantitative ladder (1kB, Eppendorf), were analyzed by electrophoresis in 1,2% (w/v) agarose gels. The gels were stained with ethidium bromide and visualized under UV-light (Gel Doc XR, Bio-Rad, Munich Germany).

DGGE and phylogenetic affiliation

DGGE was performed with the Ingeny Phor-U system (Ingeny, Goes, Netherlands), using the slightly modified protocol described by Brinkhoff & Muyzer (Brinkhoff & Muyzer 1997). After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes, Inc.) and documented using the Gel Doc XR system (Bio-Rad, Munich, Germany). Prominent bands were excised and transferred to sterile Eppendorf caps using an ethanol-sterilized scalpel. Fifty µl of water (molecular grade, Eppendorf, Germany) were added, samples were incubated at 4°C overnight for resuspension of cDNA and finally stored at –20°C.

The banding patterns and relative quantity of bands were analyzed using the software Gel Compar II, version 2.5 (Applied Maths, Kortrijk, Belgium). Cluster analysis in order to compare banding patterns was done band based on presence-absence data with the binary Dice coefficient for similarity measurement of bands and the dendogram type UPGMA (**U**nweighted **P**air **g**roup **M**ethod using **A**rithmetic averages). The normalization to the same standard of both the NA and PA gels allowed a joint cluster analysis of both assemblages.

Excised bands were sequenced by JenaGene (Jena, Germany) using the primer pair 358f and 907rM. For all sequences, at least 350 bp were analysed with the software SeqMan.

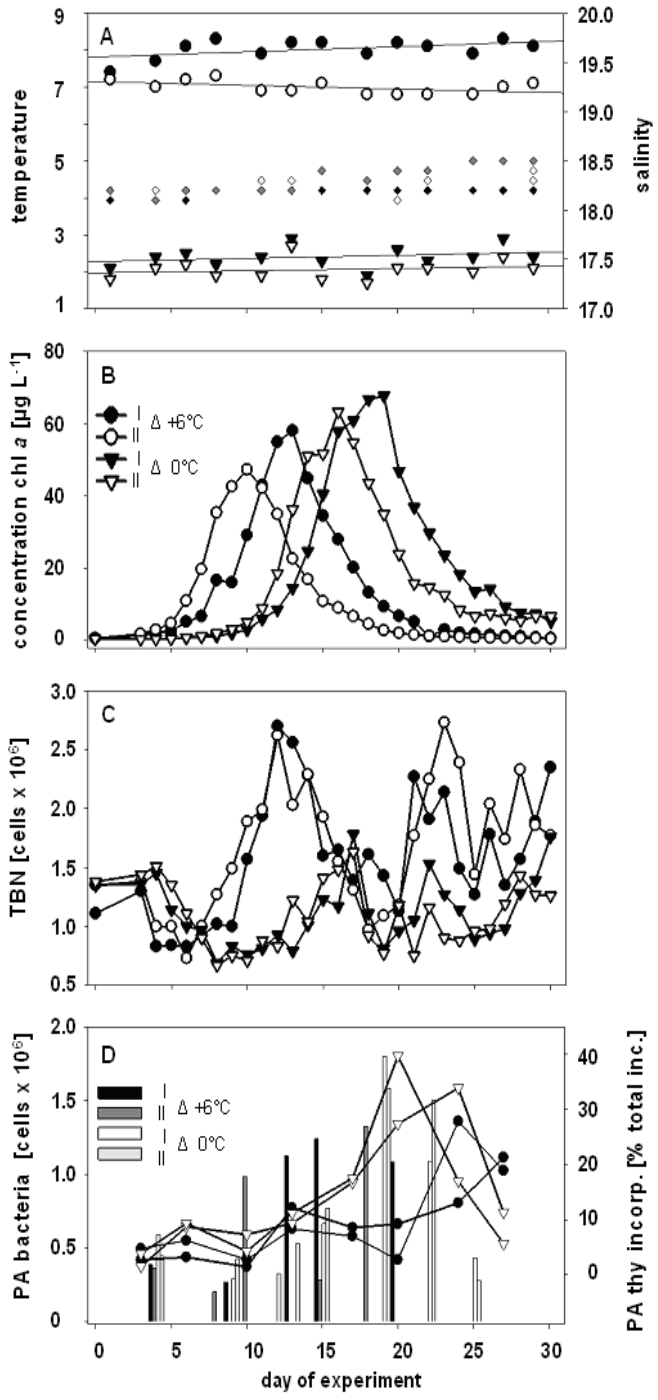
Phylogenetic affiliation was determined by using the BLAST function of the NCBI server (http://www.ncbi.nlm.nih.gov) to perform comparison with the data available in GenBank, where the sequences obtained in this study are filed under the accession numbers EU068005 – EU068025.

CARD-FISH analysis

After fixation with glutardialdehyde (4% w/v) for one hour, 40 ml water were first filtered through 3.0 µm and subsequently through 0.2 µm polycarbonate filters (diameter 47mm, Osmonics, MN, USA) in order to distinguish between particle-attached and not-attached bacterial cells. Until further processing, filters were stored in the dark at -80°C. CARD (**ca**talysed **r**eporter **d**eposition)-FISH was performed according to Sekar et al. (Sekar et al. 2003) with the following modifications: After the agarose treatment, filter snips were dried with the surface upward on object slides and covered with 96% ethanol before being peeled off again. Permeabilisation, hybridisation and the actual CARD (Grote et al. 2007) were performed in 1.5 ml reaction tubes (Eppendorf), containing six filter snips at most per step. For characterization of the bacterial community, the following horseradish peroxidase probes (Biomers.net, Ulm, Germany) were applied: EUB 338-I-III (targeting most ***Eubacteria*****,** (Amann et al. 1990, Daims et al. 1999), ALF968 (targeting most **α-*proteobacteria*,** (Neef et al. 1999))**,** BET (targeting most β-*proteobacteria*, (Manz et al. 1992)), GAM42a (targeting most γ-*proteobacteria*, (Manz et al. 1992)), CF319 (targeting many groups belonging to the *Bacteroidetes* group, (Manz et al. 1996)). The EUB antisense probe NON338 (Wallner et al. 1993) served as a negative control. After hybridization and CARD, filter snips were counterstained with Vectashield-mounting solution with DAPI (1 µg mL^{-1}). Enumeration of DAPI-positive cells (between 500 and 1.000) and hybridisation signals were counted in a minimum of 15 fields (1000x magnification), using epifluorescence microscopy (Axioskop, Zeiss, Germany).

Results

Abiotic parameters



In all four mesocosms, salinity ranged from 18.1 to 18.7 PSU during the sampling period (Fig.1A). Temperatures lay between 1.8 and 2.9 °C in the cold and 6.9 and 8.3 °C in the warm mesocosms (Fig.1A). The parallel tanks of the same treatment (P1; P2) exhibited a systematic temperature difference of an average of 0.4 °C in the warm and 1.1 °C in the cold tanks, caused by different positions of the tanks in the climate chambers. From the measured inorganic nutrients (NO_3^-, NH_4^+, PO_4^{3-}, $(SiOH)_4$; data not shown), NH_4^+ reached the detection limit first with NO_3^-, PO_4^{3-} and $(SiOH)_4$ following four days later. The decline of all four components occurred earlier with increasing temperatures, resulting in a time lag of up to seven days between warmest and coldest treatments until concentrations had reached detection limits.

Fig.1: (A) Temperature and salinity progression over experiment. Circles represent temperature in warm, triangles in cold mesocosms; salinity [PSU] is shown in diamonds with black and dark grey representing warm, white and light grey representing cold tanks. (B) Chlorophyll *a* concentration for both parallels at warm and cold temperatures over algal bloom (legend applies also for C and D), (C) total bacterial numbers for both parallels in warm and cold tanks over experiment, (D) dynamics of particle-attached [PA] bacterial cell numbers (bars) for both parallels over algal bloom; development of PA bacterial production as percentage of total thymidine incorporation (curves).

Dynamics of phyto- and bacterioplankton and bacterial activity

In both parallels and at both temperatures, distinct phytoplankton blooms were observed, with chl *a* concentrations being slightly higher at cold temperatures, where they reached 67.7 (parallel I) and 63.3 µg L^{-1} (parallel II, Fig.1 B). Values at warm temperatures exhibited maxima of 58.0 and 47.3 µg L^{-1}, respectively. In bloom onset and timing of maximum chl *a* concentrations, a time lag occurred between temperature treatments as well as parallel tanks. Maximum chl *a* concentrations were reached after 9 (parallel I) and 12 days (parallel II) at warm, and after 15 and 19 days at cold temperatures. In warm tanks, the set off of algal growth took place after five days and therefore four days earlier than in cold treatments, where phytoplankton growth set in at day nine. In all mesocosms, algal blooms were dominated by diatoms with *Skeletonema costatum* being the most abundant species, whereas flagellates played only a minor role (data not shown).

Cell counts of the NA bacterial fraction at warm temperatures ranged from 0.73 to 2.62 x 10^6 cells mL^{-1}. In cold mesocosms, these values ranged between 0.67 to 1.78 x 10^6 cells mL^{-1} (Fig. 1 C). The dynamics of bacterial abundance revealed a similar trend for both treatments and parallels. From similar starting values between 1.11 and 1.38 x 10^6 cells mL^{-1}, cell numbers declined during the first days of the experiment to increase again up to a first peak concurrent with the phase of the phytoplankton peak. This stage was followed by a distinct decline of cell numbers during the initial algal degradation phase and a second peak parallel to the decline of the phytoplankton bloom. These dynamics were more pronounced and took place earlier at warm temperatures,where also higher cell numbers were detected than in the cold treatments. Furthermore, the time lag we found between parallels at same temperatures in the algal dynamics was not detected in bacterial development (Fig.1C).

Particle-attached bacteria first decreased during the exponential phase of the algal bloom and rose again during the break down of phytoplankton at all temperatures (Fig.1D). Only in parallel II at cold temperatures did a continuous increase of the bacterial PA fraction over the entire algal bloom take place. Maximum counts were 2.32 x10^6 and 2.52 x10^6 cells mL^{-1} at warm and 3.53 x10^6 and 2.67 x10^6 cells mL^{-1} at cold temperatures. In the warm tanks, minimum counts were slightly lower (1.42 and 1.19 x10^6 cells mL^{-1}) than in the cold mesocosms (1.7 and 1.67 x10^6 cells mL^{-1}). In contrast to cold temperatures, particle-attached bacteria in the warm tanks were in the same range as in the NA-fraction. In the cold mesocosms, however, PA bacteria were almost constantly higher than not-attached cells.

The share of PA bacteria in the total bacterial secondary production rates mirrored the dynamics of PA cell counts only in the cold tanks where percentages of PA thymidine incorporation continuously increased and peaked at the same time as particle-attached cell abundance (Fig.1D). At warm temperatures, the increase in relative PA production occurred slightly later. The relative contribution of PA bacteria to total bacterial production reached maxima of 33.9 and 39.7% in warm (parallels I and II) and 27.8 and 21.3%, in cold tanks..

CARD-FISH results

For a quantitative assessment of the bacterial community composition by CARD-FISH, we chose three points of the overall phytoplankton development (middle of the exponential phase, peak of bloom, end of degradation phase) and analysed the samples from each parallel and temperature treatment taken at this point. Thus, bacterial communities from the same stage of the algal bloom were compared with each other. Since the bloom developed differently at both temperatures, this resulted in different dates of analysis for each tank.

The percentage (% DAPI) of NA cells hybridised with the probe EUB338 ranged from 57.4 to 65.1% (average of 61.3%) in warm and from 56.2 to 62.3% (average of 59.3%) in cold tanks, respectively. In the PA fraction, EUB positive cells reached average values of 72.3% DAPI at warm and 73.4% DAPI at cold temperatures (data not shown). In the NA fraction, α-*Proteobacteria* were most abundant with maxima of 9.3 (P2, t_2) and 11.5 % DAPI (P1, t_2) at warm and cold temperatures, respectively (Fig.2A). At all three cardinal points, counts for α-*Proteobacteria* were higher in cold tanks. β-*Proteobacteria* revealed lowest group-specific counts with maxima of 4.0 (P1) and 4.6 %DAPI (P2), both at t_1 (Fig.2B). The counts of γ-*Proteobacteria* were characterized by a high variability between the two parallels at cold temperatures, but showed higher % DAPI in cold treatments at average (Fig.2C). At both temperatures, proportions of *Bacteroidetes* revealed an increase with the progressing algal bloom, which was more pronounced in warm tanks where 14.79 (P1) and 12.09 % DAPI (P2) were reached at t_3 (Fig.2A, D).

In the PA fraction, *Bacteroidetes* dominated the community, reaching maxima of 50.0 (P2, t_2) and 41.4 %DAPI (P1, t_3) at cold and warm temperatures respectively (Fig.2H). Lowest counts were again found for β-*Proteobacteria* with maxima of 3.2 % DAPI (P2, t_3) in warm and 5.9 %DAPI (P1, t_1) in cold tanks (Fig.2F). Unlike in the NA fraction, PA α-*Proteobacteria* ranged within similar percentages at both temperatures most of the time (Fig.2E). Cell counts of γ-*Proteobacteria* revealed a trend of higher percentages in cold tanks

at all cardinal points except for P2 at t_3 , reaching early maxima of 13.6 (P2, t_1) at warm and 17.3 % DAPI (P1, t_1) at cold temperatures (Fig.2G).

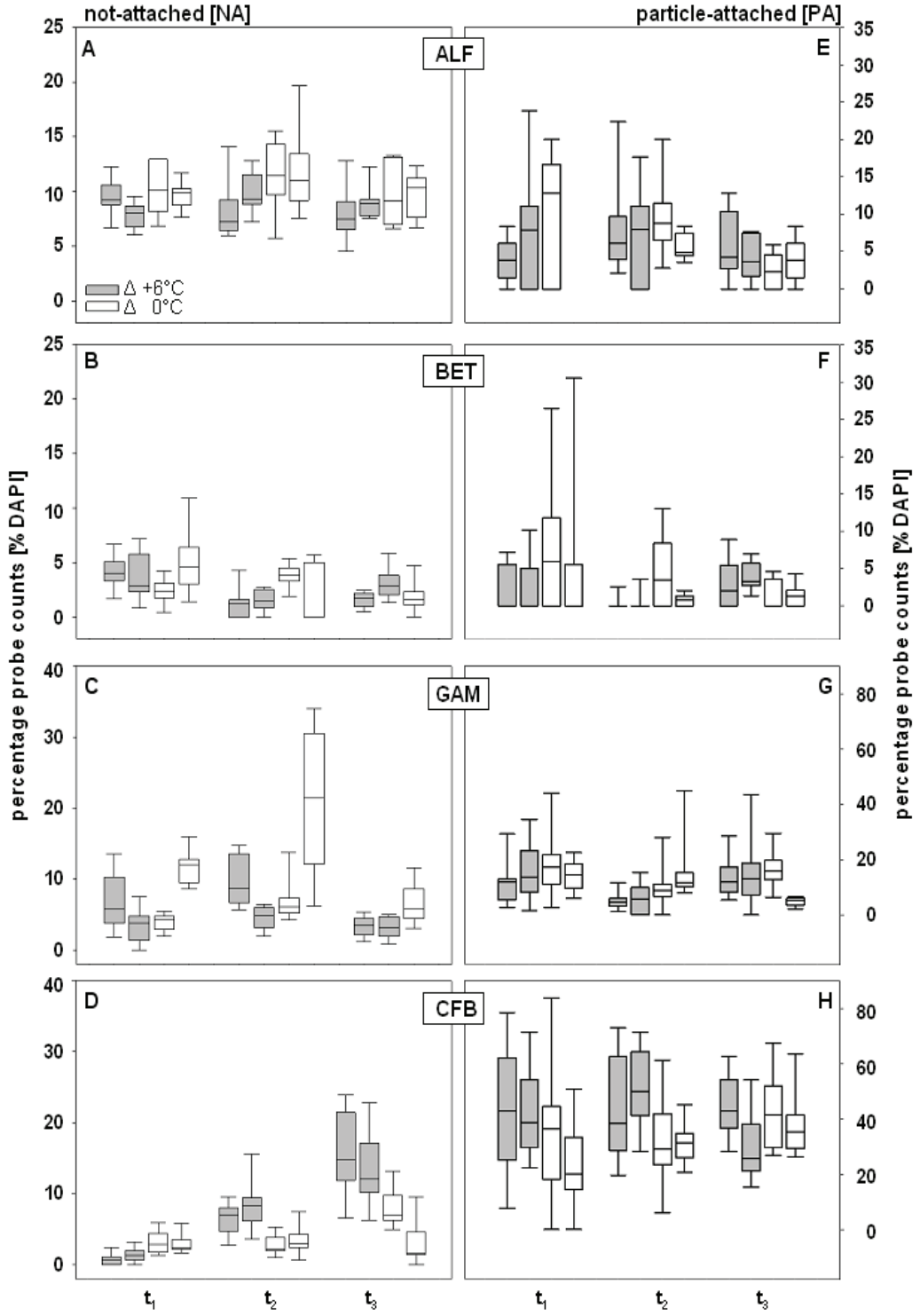


Fig. 2: CARD-FISH counts of bacterial groups α- (ALF), β- (BET), γ-*Proteobacteria* (GAM) and *Bacteroidetes* (CFB) for NA [(A), (B), (C) and (D)] and PA fraction [(E), (F), (G) and (H)]. Diagrammed are both parallels at three cardinal points of the algal bloom (t_1 = exponential phase, t_2 = peak, t_3 = degradation phase). All values of single counting grids were included in calculation to display median values and counting variability. Box-Whisker-plots show the $25^{th}/75^{th}$ percentile (box), the mean (solid line) and the $5^{th}/95^{th}$ percentile (whiskers).

PA-fraction, α-*Proteobacteria* decreased on average over the bloom in both treatments, a

trend that was more pronounced at cold temperatures (Fig. 2E). The dynamics for β-and γ-*Proteobacteria* differed with temperature, reaching a minimum at the peak of the bloom and increasing again afterwards in the warm tanks, but declined in the cold treatments over the algal bloom (Fig.2 F, G). *Bacteroidetes* counts reached a maximum at the peak of the bloom and decreased afterwards at warm, but showed an ongoing increase at cold temperatures (Fig.2H).

Not-attached α- and γ-*Proteobacteria* showed the same average dynamics at both temperatures, revealing maximum values at the peak of the bloom and decreasing towards the degradation phase (Fig.2A, C). Counts of NA β-*Proteobacteria* differed with temperature, showing similar dynamics as in the PA community (Fig.2B). Average proportions of *Bacteroidetes* increased over the course of the phytoplankton bloom at both temperatures, a trend that was more pronounced in warm treatments (Fig.2D).

DGGE fingerprints, phylogenetic affiliation and cluster analysis

As for the CARD-FISH analysis, we chose several points of the overall algal development (start of the bloom, middle of the exponential phase, peak of bloom, middle and end of degradation phase) at which samples from each parallel and temperature treatment were taken and analysed. Since the algal blooms lasted longer at cold temperatures, two points were analysed during exponential and degradation phase instead of one in the warm treatments. With the applied primer set specific for *Bacteria*, the fingerprint of the NA fraction (Fig. 3A) revealed between 16 and 20 at Δ +6 °C and between 14 and 21 different bands at Δ 0 °C. For the PA fraction, 12 to 20 bands were detected in the warm and 11 to 20 in the cold treatments (Fig. 3B). Though there were variations in intensity, several bands persisted throughout the entire algal bloom in both fractions, and most bands occurred at some point of time during the experiment regardless of fraction and temperature.

For identification of dominant ribotypes, 9 bands from the PA and 12 bands from the NA fraction were excised from the DGGE gels and sequenced (marked by arrows in Fig.3). The results revealed a strong bias of chloroplast 16S rRNA gene fragments (Tab.1). In the PA fingerprint, four bands were assigned to diatoms (AS06-1PA/-10PA; AS06-2PA; AS06-5PA). Two chloroplast-derived bands (AS06-12NA, AS06-19NA) were detected in the NA fingerprint and two ribotypes (AS06-20NA, AS06-24NA) could be assigned to *Prymnesiophyceae*.

In the PA fraction, almost all bacteria-derived ribotypes were assigned to the *Bacteroidetes*-phylum (AS06-6PA, AS06-8PA, AS06-9PA, AS06-4PA affiliated with *Flexibacter litoralis*). Only one band (AS06-7PA) was related to the β-*proteobacteria*. The ribotypes AS06-4PA and AS06-9PA (Fig.4B) were present at warm and cold temperatures, albeit not over the entire algal bloom. At both treatments and parallels, the bands AS06-6PA and AS06-18PA persisted at each point of sampling. The band AS06-7PA which affiliated with *Hydrogenophaga pseudoflava* (similarity 96%) and could only be detected at cold temperatures, appeared during the degradation phase of the algal bloom. No band solely present at warm temperatures was found.

Within the NA fingerprint, two ribotypes (AS06-15NA; AS06-16NA) were affiliated with the *Bacteroidetes* phylum; AS06-22NA was closest related to the same uncultured β-*proteobacteria* isolate as AS06-7PA from the PA fraction. Comparison of the sequences of AS06-NA21 and AS06-7PA using BLAST showed 100% similarity, suggesting that both ribotypes are identical.

Most of the bacterial-derived NA ribotypes were identified as α-*Proteobacteria*, with AS06-14NA/-23NA and AS06-18NA belonging to the *Roseobacter* clade affiliated RCA-cluster (Selje et al. 2004). The number of persistent bands of bacterial origin was higher in the NA than in the PA fraction (Fig.4 A). The bands AS06-14NA, AS06-15NA, AS06-21NA, AS06-23NA and AS06-22NA occurred at both temperature treatments and over the entire phytoplankton bloom. The NA bacterial community did not exhibit any bands solely present at warm or cold temperatures.

To back up the assumption that same band positions represent same ribotypes, we did a spot check by sequencing. For each fraction, one band which was present at the same gel position at both temperatures was randomly selected (AS06-23NA, AS06-14NA, AS06-6PA, AS06-8PA), excised and sequenced. In the NA-fraction, both bands were affiliated with the uncultured clone RCA 11-5 of the RCA-cluster. In the Pa-fraction, both ribotypes were similar to the same uncultured *Bacteroidetes*-bacterium.

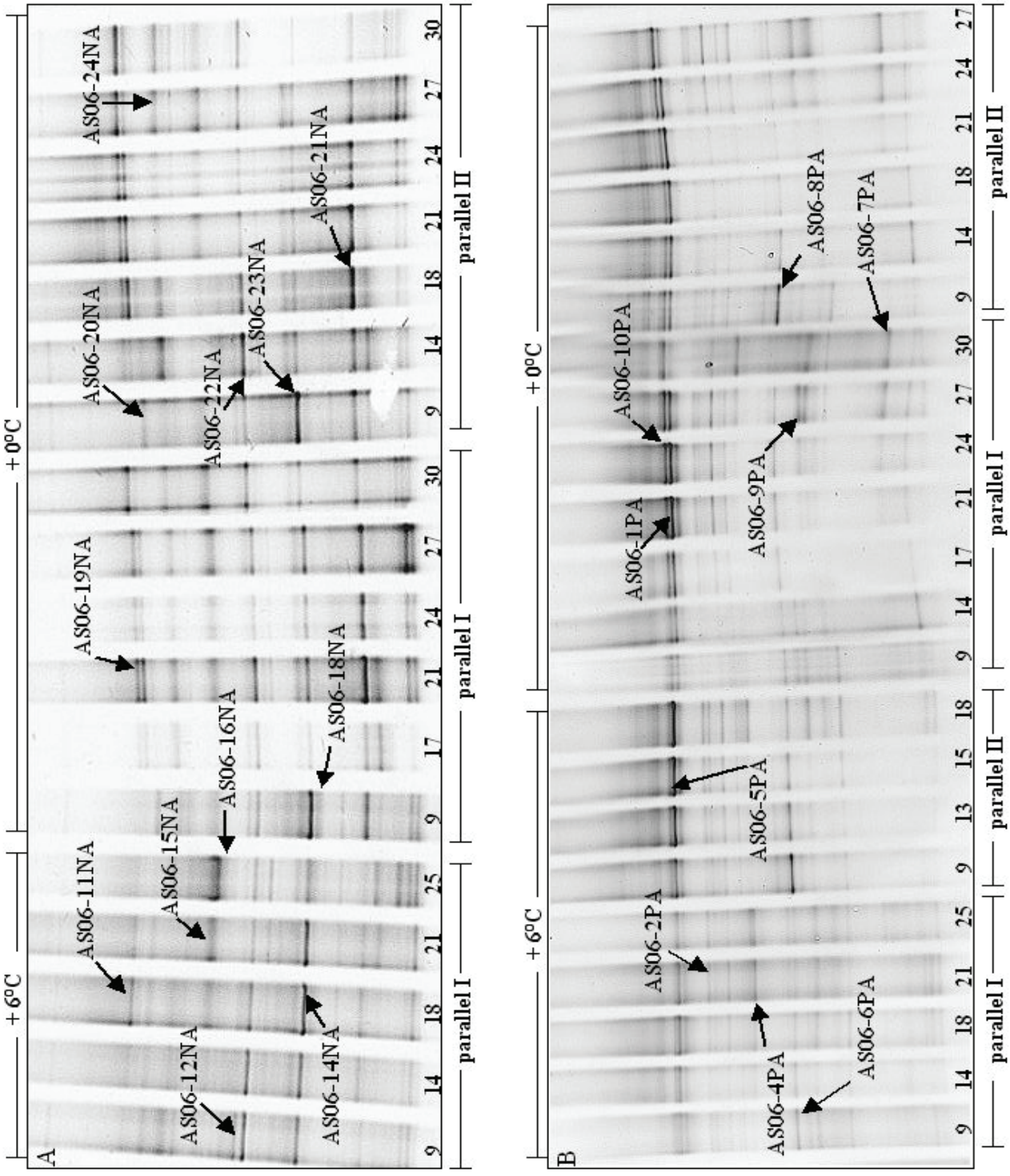


Fig. 3: DGGE fingerprints of (A) not-attached [NA] and (B) particle-attached fraction for several points of time over phytoplankton bloom at cold [Δ 0°C] and warm [Δ +6°C] temperatures. Arrows mark excised and sequenced bands (see Tab.1 for phylogenetic band affiliations).

Table 1: Band identification (band ID) and phylogenetic affiliation of excised bands for the bacterial not-attached [NA] and particle-attached [PA] fraction at the temperature extremes, along with their accession number. Further given are closest (cultivated) relative and the similarity to them.

band ID	phylogenetic class	closest published relative	similarity	accession no.
AS06-1-PA	chloroplast	*Lauderia borealis*	98%	AJ536459
AS06-2-PA	chloroplast	*Thalassiosira nitzschioides*	95%	AJ536454
AS06-4-PA	*Bacteroidetes*-clade	*Flexibacter filoralis*	98%	AB078056
AS06-5-PA	chloroplast	*Skeletonema pseudocostatum*	99%	X82155
AS06-6-PA	*Bacteroidetes*-clade	unc. *Bacteroidetes* bacterium	95%	AB266606
AS06-7-PA	β-*proteobacterium*	unc. β-*proteobacterium* isolated DGGE band	99%	DQ080926
AS06-8-PA	*Bacteroidetes*-clade	unc. *Bacteroidetes* bacterium isolated DGGE band GWS-e8-FL	95%	AY274242
AS06-9-PA	*Bacteroidetes*-clade	unc. *Bacteroidetes* bacterium clone Flo-32	98%	AY684351
AS06-10-PA	chloroplast	*Lauderia borealis*	98%	AJ536459
AS06-11-NA	*Bacteria*	unc. bacterium clone 245b3	97%	EF460102
AS06-12-NA	chloroplast	unc. cryptomonad OM 283	96%	U70724
AS06-14-NA	α- *proteobacterium*	unc. *Rhodobacteraceae* bact clone RCA11-5/RCA10-2	99%	DQ489286
AS06-15-NA	*Bacteroidetes*-clade	unc. *Flavobacteria* bacterium clone MS024-3C	100%	EF202327
AS06-16-NA	*Bacteroidetes*-clade	unc. *Bacteroidetes* bacterium clone	83%	AY580734
AS06-18-NA	α-*proteobacterium*	unc. *Rhodobacteraceae* clone RCA11-5	96%	AY165488
AS06-19-NA	chloroplast	*Lauderia borealis*	99%	AJ536459
AS06-20-NA	eukaryote	unc. Phototrophic eukaryote clone GL2-30	90%	EF215744
AS06-21-NA	β-*proteobacterium*	unc. *Bacteroidetes* bacterium isolated DGGE band GWS-e8-FL	99%	AY274242
AS06-22-NA	α-*proteobacterium*	unc. α-*proteobacterium* isolate	98%	DQ911784
AS06-23-NA	α-*proteobacterium*	unc. *Rhodobacteraceae* bact. clone RCA-H28	99%	DQ489286
AS06-24-NA	eukaryote	unc. Phototrophic eukaryote clone GL2-30	90%	EF215744

Though we did detect cells of the subclass γ-*Proteobacteria* by CARD-FISH among the particle-attached as well as the not-attached bacterial assemblages, we found no affiliated sequences in neither the PA- nor in the NA-fraction.

Comparison of banding patterns, excluding the bands derived from chloroplasts, by cluster analysis revealed marked differences between the not associated and particle-associated fraction, suggesting a different structure of the two communities (Fig.4). From the two subclusters that formed within the NA cluster, one solely contained samples taken at cold temperatures. The second one, however, was formed of samples from both temperatures with even one branch containing banding patterns originating from cold and warm treatments. In contrast, the two marked subclusters within the PA-cluster included only samples from one temperature treatment, except for the two earliest samples from the cold treatments (temp. 0°C, parallel I and II, day 9) which fell into the 'warm' subcluster.

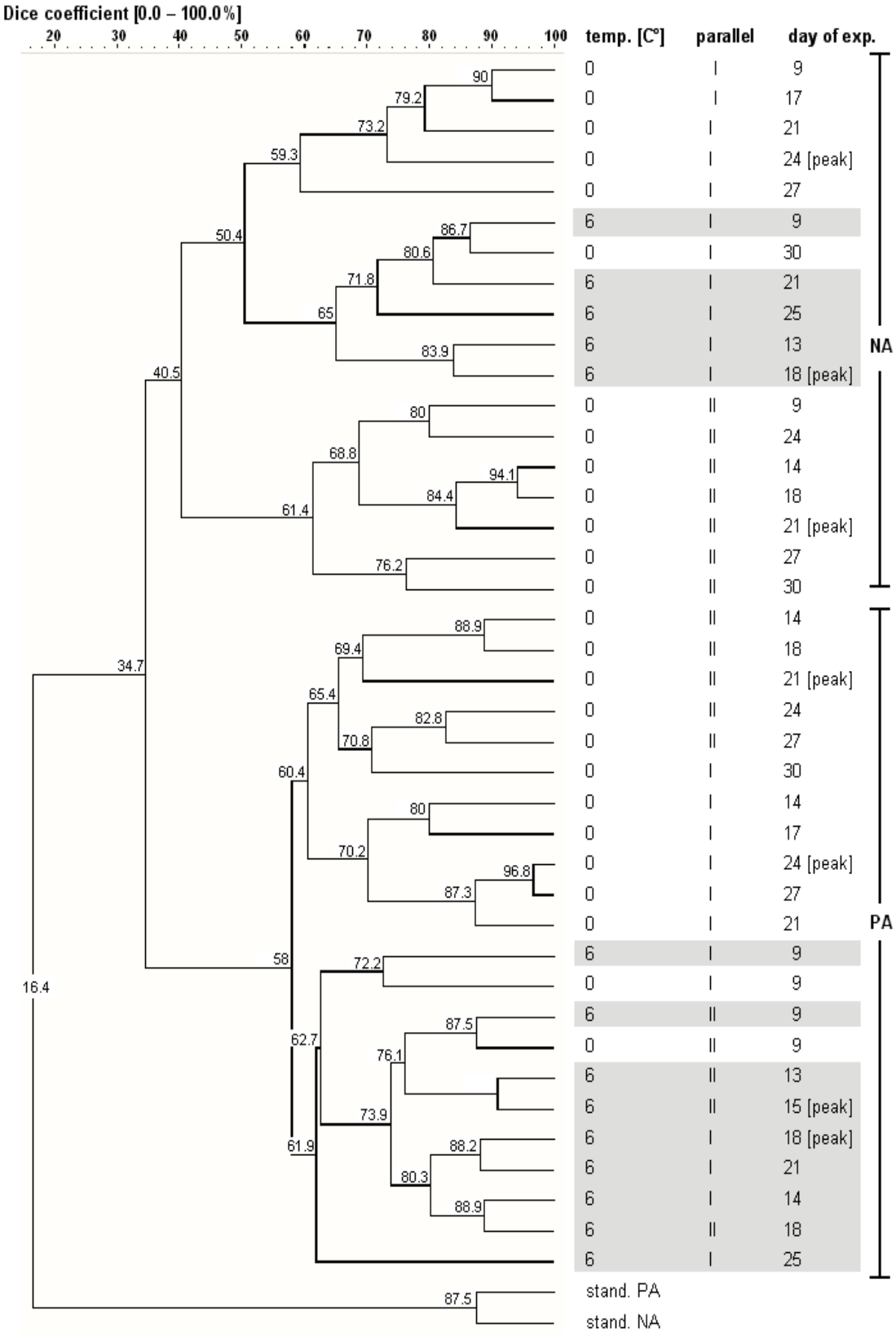


Fig. 4: Curve based cluster analysis (UPGMA = unpaired group method of analysis) of DGGE fingerprint banding patterns (Fig.3) for two parallels (I /II) of not-attached [NA] and particle-attached [PA] fraction at cold [Δ 0°C] and warm [Δ +6°C] temperatures. Numbers in branches stand for calculated cophonetic correlations of each branching; patterns from warm samples are shaded gray.

Discussion

The experimental design we used allowed us to successfully induce and analyse a phytoplankton bloom as well as the parallel developing bacterial community under early spring conditions in all four mesocosms. Though mainly the tanks of the warm treatment notedly varied in temperature, probably due to the mode of function of the climatic chambers, we were able to simulate a temperature gradient within the range predicted by the IPCC (Houghton & others 2001). Aside from the chl *a* concentration, the temporal dynamics of the dominating *Skeletonema costatum* (data not shown) were the only parameters affected by the temperature differences between the parallel tanks (Fig.1B, Fig.2A and B). Numbers of the most abundant diatom and flagellate species (Fig.2A and B) did not show a time lag in bloom development between the two parallels of the same temperature, and neither did TBN (Fig.1C). This indicates that the development of the dominating algal species *Skeletonema costatum* was affected by the temperature variations between parallels (Fig. 1 A), which was in turn mirrored by fluorescence measurements of chl *a*. Since phytoplankton composition was very similar in the Δ 0°C and the Δ +6°C treatment (Fig.2), potential variations of the BCC between temperature treatments did not result from different phytoplankton communities, an effect that already was observed by Grossart et al. (Grossart et al. 2005). Considering these results, we assume similar algal-derived conditions for the development of the bacterial communities at both temperature extremes.

The bacterial community structure in general as well as the differences between NA and PA assemblages we found by CARD-FISH and DGGE analyses (Fig.3, 4) are supported by the findings from several other studies (DeLong et al. 1993, Bidle & Fletcher 1995, Acinas et al. 1999). *Bacteroidetes*, acknowledged participants in the break-down of complex polymers (Reichenbach 1992, Kirchman 2002), are known to dominate the PA (DeLong et al. 1993, Bidle & Azam 2001, Moeseneder et al. 2001), whereas α- and γ-*Proteobacteria* typically prevail in the NA fraction (Böckelmann et al. 2000, Bouvier & del Giorgio 2002, Simon & Tilzer 2002, Selje & Simon 2003, Castle & Kirchman 2004). These findings were supported by our sequencing results and those of several other authors (Riemann et al. 2000, DeLong et al. 1993, Acinas et al. 1999, Crump et al. 1999, Selje et al. 2004). The lack of DGGE-ribotypes affiliated to γ-*Proteobacteria*, despite respective CARD-FISH signals in both bacterial fractions, is a phenomenon which has already been encountered and elaborately discussed by several authors (Selje & Simon 2003, Castle & Kirchman 2004).

Regarding these general findings we are confident that our experimental design provided 1) suitable conditions as natural as possible to analyse the changes within basic functions and principals of the coupling between algae and bacteria at elevated temperatures and 2) resulted in a bacterial community that was not artificial but showed the expected structure on the group as well as the species level.

General algae-bacteria dynamics

Quantitative data from chl *a* concentration and bacterial cell counts revealed the well-known coupling between the development of phyto- and bacterioplankton dynamics at both warm and cold treatments (Fig.2B, C, D). The first peak of TBN most likely mirrored the increase of algal exudates during the exponential phase of phytoplankton growth e.g., an effect also described by other authors (Bell & Kuparinen 1984, Karner et al. 1992). The second increase in TBN as well as the growing numbers of PA bacterial numbers were most probably a result of the breakdown of the algal bloom (Pedros-Alio & Brock 1983, Simon 1987) that occurred after depletion in inorganic nutrients. Particle-associated bacteria are known for their relevance in the collapse of phytoplankton blooms by degrading organic material (Hoppe et al. 1993, Middelboe et al. 1995 , Becquevort et al. 1998), which in turn is again available for use by the NA bacterial community. The increasing PA bacterial secondary production over the progressing bloom, especially at cold temperatures, substantiates the assumption of direct bacterial utilisation of algal produced organic matter and therefore a close functional coupling of phyto- and bacterioplankton in our experiment. Furthermore our CARD-FISH results showed that not only abundance but also the proportions of bacterial subgroups changed with the progressing algal bloom in both fractions (Fig. 4). Especially the counts of NA Bacteroidetes markedly increased with the duration of the bloom (Alderkamp et al. 2006, Rink et al. 2007), indicating that during growth and degradation phase of the phytoplankton blooms complex and polymeric DOC was released which can be utilized by members of the Bacteroidetes due to their reported capability to hydrolyze polymers (Kirchman 2002, Bauer et al. 2006).

In contrast to other studies (Riemann & Winding 2001, Schäfer et al. 2001, Pinhassi et al. 2004), our genetic fingerprint analysis did not suggest qualitative shifts in neither the NA nor the PA fraction with progress of the algal bloom. This could point to either a rather uniform substrate background throughout the algal bloom or indicate that the universal bacterial primer set was not specific enough to ensure the amplification of species that were

subject to shifts caused by the progress of the phytoplankton bloom. Rink et al. (Rink et al. 2007) examined the effects of a phytoplankton bloom on bacterial communities in a coastal ecosystem and found only weak shifts with the universal *Bacteria*-targeted primer set but were able to show clear changes with the more specific primer sets targeting *Roseobacter* and *Bacteroidetes*. It is therefore likely that the bacterial communities in our tanks underwent compositional changes during the phytoplankton blooms which were not detected by the primer set we used.

Temperature influence on the bacterial community composition

The effect of ambient temperature on the BCC has been analysed in several *in situ* studies, yet their results do not suggest a discernable impact of ambient temperature on the bacterial community structure (Schauer et al. 2003, Zwisler et al. 2003, Henriques et al. 2006). In contrast to those studies which referred exclusively to the not-attached bacterial fraction, our analyses go beyond those mentioned above. We not only included the particle-attached community in our study, but also propose that ambient temperatures actually do influence the BCC in our mesocosms, apparently affecting the NA and PA bacterial community to different extents.

On a group-specific level, shifts within the BCC in our mesocosms were restricted to certain bacterial groups. Comparison of our CARD-FISH results from the two temperatures indicated that differences which applied to all tested points of time were limited to PA and NA *Bacteroidetes* as well as NA α-*Proteobacteria*, suggesting that temperature had an effect on these two bacterial subgroups. The remaining variations in both NA and PA fraction were mainly found at the peak of the algal bloom (t_2) and always limited to two of the three tested points of time. Though we are not able to exclude temperature as a directly determining factor in these cases altogether, we assume that differences applying all tested stages of the bloom could also point to other sources of influence, for example affects mediated by algal-dependent organic matter supply. It has been shown in several studies that alterations in DOC supply during the course of an algal bloom result in shifts within the bacterial community composition (Fandino et al. 2001, Yager et al. 2001, Larsen et al. 2004). Moreover, organic matter released by phytoplankton and resulting from algal degradation might qualitatively vary with temperature, resulting in distinct quantitative developments in certain bacterial subgroups due to different organic resources (Wohlers et al., unpublished). Temperature therefore might have affected the bacterial community composition indirectly.

We are aware that we missed members of e.g. δ-, ε-*Proteobacteria* and *Actinobacteria* in our CARD-FISH analysis, which most likely is the main reason that not even half of the detected NA *Bacteria* could be assigned to the groups we were targeting. Since PA bacterial activity increased over the course of the algal blooms (Fig. 2D), most likely due to enhanced degradation of organic matter, PA cells probably were more active and bigger and than those in the surrounding water (Grossart & Simon 1998, Zwisler et al. 2003, Grossart et al. 2007). This might have resulted in a general underestimation of less active and smaller NA cells, the high sensitivity of the CARD-FISH method notwithstanding. Still, the proportions of EUB338 positive cells of our studies go along with the finding of other authors (Alonso-Saez et al. 2007, Rink et al. 2006, unpublished), which makes us confident that our CARD-FISH hybridisation efficiency yielded reliable results.

Though the visual impression of our genetic fingerprints indicated a rather uniform BCC for both fractions, temperature-dependent comparison of our DGGE data using cluster analysis revealed temperature-linked clustering of different extent in the two fractions we analysed (Fig.4, 5A, B). Within the three subclusters of the NA assemblage, the groupings that formed hinted that unique bacterial communities developed at particular temperatures. Still, the similarity between the BCCs from the first parallels of coldest and warmest treatments did not suggest distinct temperature-related differences. Though our CARD-FISH results imply shifts within the composition of the NA fraction between temperature treatments, we found merely a weak respective signal on the species level.

In contrast, with the exception of only two samples, both originating from the algal bloom onset when differences between treatments apparently were not as pronounced yet, the PA bacterial fraction exhibited a clear splitting into two temperature-linked subclusters, confirmed by the finding of the only prominent temperature-specific ribotype found in the PA fraction. The PA band AS06-7PA, which was affiliated with the β-*Proteobacteria*, only occurred at cold temperatures. The closest cultivated relative of AS06-7PA was *Hydrogenophaga pseudoflava* (sim.: 96%), a freshwater β-*Proteobacterium* which has been found from temperate to Antarctic regions (Rutter & Nedwell 1994), though it shows a range of optimum temperature from about 2 to 27°C (Reay et al. 1999). It is known that β-*Proteobacteria* are part of the PA bacterial community in brackish and fresh water systems (Selje & Simon 2003, Böckelmann et al. 2000), contributing to the decomposition and release of aggregate-bound amino acids (Schweitzer et al. 2001) which might explain why AS06-7PA only appeared after the algal peaks and increased in intensity during the late bloom (Fig.4B).

In contrast to that, the NA phylotype (AS06-NA21) also affiliated with *Hydrogenophaga pseudoflava* (sim.: 96%) occurred at both temperatures (Fig.4A), indicating the active presence of this β-*Proteobacterium* -related ribotype in both fractions , which apparently was only affected by temperature in its degrading function of organic particles.

The stimulating effect of rising temperature on the activity of bacteria is well documented (Pomeroy & Wiebe 2001, Kirchman et al. 2005, López-Urrutia & Morána 2007). To determine whether these alterations in bacterial activity are accompanied by changes in the structure of bacterial assemblages, we set out to analyse the bacterial community composition during phytoplankton spring blooms at two different temperatures. We were able to show that rising temperatures directly affected the bacterial community composition in our mesocosms. Despite several dominating bands persisting throughout the entire experiment irrespective of fraction and treatment, DGGE fingerprinting revealed two clearly temperature-linked PA bacterial communities. Furthermore, CARD-FISH analysis, though only carried out for the major groups α-, β- and γ-*Proteobacteria* as well as *Bacteroidetes*, exhibited quantitative temperature-related differences for some of the tested groups. These findings support our over all results which suggest that already an increase of 6°C in temperature does have an influence on the present bacterial community, with the PA fraction apparently being more affected than not-attached bacteria. Using more specific primers instead of a general *Eubacteria* primer-set in future studies might achieve a higher resolution of genetic fingerprinting and therefore grant a more detailed insight into the effect of rising temperatures on the bacterial NA fraction also on a phylogenetic level.

III. Shifts in the bacterial community composition during an induced algal bloom under the influence of rising temperatures and different nutrient regimes

Abstract

Indoor bottle experiments were conducted to examine the combined effect of inorganic nutrient concentrations and rising temperatures on the functional phytoplankton-bacteria coupling as well as the bacterial community composition (BCC) during early spring bloom conditions. In these experiments, axenic cultures of *Skeletonema costatum* were incubated in aged seawater from the Baltic Sea at three different temperatures (4°C, 8°C and 12°C) und exposed to two different nutrient regimes of N:P= 5 and N:P=30. During the course of the induced bloom of *Skeletonema costatum,* bacterial production was assessed by ^{3}H-leucine incorporation, the composition of the non-attached (NA) as well as the particle-attached (PA) bacterial assemblage was examined by the PCR-based genetic fingerprint method denaturating gradient gel electrophoresis (DGGE) as well as the catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) method. Several indications for synergy between nutrient concentrations and temperature were found. The analysis of the bacterial assemblage by cluster analysis of the genetic fingerprints pointed to a temperature effect on the bacterial community composition during the late stage of the algal bloom which was more pronounced for PA bacteria and at higher N-concentrations. Furthermore, the sequencing of excised prominent bands revealed the highest abundance of temperature-specific ribotypes in the high-N PA-fraction. High-N leucine incorporation as a proxy for bacterial production was markedly lower at coldest (maximum values of 2,6 x10^3 pM/h) compared to medium and warm temperatures (maximum values of 7,1 and 5.3 x10^3 pM/h, respectively). This study therefore not only substantiates the results of a previous experiment which pointed at a more pronounced temperature effect on the PA than on the NA bacterial community composition during the late stages of a phytoplankton bloom. It also suggests that an accurate assessment of the influence of temperature on the functional coupling of phyto- and bacterioplankton in the course of global warming requires taking synergetic effects between nutrient conditions and temperature into consideration.

Introduction

Within the marine biogeochemical carbon cycle, heterotrophic bacterial communities are the main degraders and consumers of autotrophically derived particulate and dissolved organic matter. Particle-attached heterotrophic bacteria are known to play a major role in the degradation of complex organic compounds, breaking them down to readily consumable dissolved organic matter which, along with other dissolved carbon sources such as algal exudates, is utilized by the non-attached fraction of the heterotrophic bacterial community (van Es & Meyer-Reil 1982, Kirchman 2002). Changes of the carbon-degrading and consuming activity of the bacterial assemblage might therefore have major effects on the marine carbon cycle. As a consequence, DOC might accumulate in the water column (Zweifel et al. 1995, Thingstad et al. 1999, Carlson et al. 2002) and the effectiveness of the degradation of organic matter, the quality of the resulting DOC and finally its bioavailability might be affected as well (Carlson et al. 2004). This could in turn considerably influence the extent of the 1) recycled carbon within the microbial loop (Azam et al. 1983), 2) refractory carbon being transported to the ocean depth and 3) CO_2 released to the atmosphere (Azam 1993). The microbial loop has enormous relevance for the function of the ocean as a carbon sink or source. It is therefore of great importance to know whether and how changes in environmental conditions affect the performance of the bacterial community in terms of activity and composition, since both aspects affect the performance of bacteria as agents within the microbial loop.

There are several environmental factors potentially influencing bacterial efficiency to break down complex organic particles as well as utilize dissolved organic carbon. The quantity and quality of the organic carbon itself, as well as ambient temperature and inorganic nutrients have been studied in the past as 'bottom-up' factors controlling bacterial growth (Pomeroy & Wiebe 2001, Baines & Pace 1991, Chrzanowski et al. 1995). Predation by heterotrophic nanoflagellates or lysis by viruses are considered 'top-down' factors regulating bacterial populations (Fuhrman & Noble 1995, Place & Cole 1996, Weinbauer & Höfle 1998). There is the notion that low temperatures limit bacterial production and activity; still there are some studies from the extreme environments of the Antarctic costal waters which suggest rapid growth of bacteria at temperatures <2° (Fuhrman & Azam 1980, Hanson et al. 1983), and some results indicate that surplus nutrients may override temperature limitation

(Nedwell & Rutter 1994, Pomeroy et al. 1991). Still, results from temperate waters indicate a close positive correlation between temperature and bacterial production and respiration (Felip et al. 1996, Pomeroy & Wiebe 2001, Kirchman et al. 2005, Lopez-Urrutia et al. 2006).

Aside from temperature, nutrients are considered to potentially affect bacterial growth. In several studies, the bioavailability of organic C and inorganic P was found to be a factor controlling bacteria (Zweifel et al. 1993, Sala et al. 2002, Thingstad et al. 2005). In a study in subarctic waters Kirchman (Kirchman 1990) found that organic N is highly stimulating marine bacterial growth. Yet it has been shown that nutrients and temperature are factors that have to be considered together to be able to describe their limiting effect on marine heterotrophic bacteria (Nedwell & Rutter 1994, Reay et al. 1999, Pomeroy & Wiebe 2001).

Not only bacterial activity is influenced by organic substrates, inorganic nutrients and temperature. Several studies could demonstrate that also the bacterial community composition shows rather fast reactions to changes in the phytoplankton community structure (Pinhassi et al. 2004, Abell & Bowman 2005, Grossart et al. 2005). These authors concluded that different algae exude qualitatively different organic matter and therefore select for a distinct structure within the bacterial community. Further results are in accordance with the notion that bioavailability of organic substrate influences the structure of bacterial assemblages, but also indicate that inorganic nutrients are of relevance as well when it comes to the bacterial community composition (Hutchins et al. 2001, Pinhassi & Berman 2003) and that even the seasonal variability of nutrient limitation has reasonable impact on structure and activity of bacterial communities (Pinhassi et al. 2006).

There are contradicting results on the effect of temperature on the composition of bacterial assemblages. While some authors were not able to associate structural changes in bacterial communities with ambient temperature (Zwisler et al. 2003, Henriques et al. 2006) or found rather stable communities over the year (Schauer et al. 2003), others could show that some environmental factors including temperature significantly affected bacterial diversity and community structure (Muylaert et al. 2002, Yannarell & Triplett 2004). Altogether these findings indicate that the same environmental factors affecting heterotrophic bacterial activity also influence the bacterial community composition. Our own results from a previous mesocosm experiment indicated that the community composition of non-associated and particle-attached bacteria during an algal spring bloom are affected to a different extent by temperature shifts (Walther et al. in prep.): during P-limited early spring conditions resembling those in the Kiel Fjord, we found that the particle-attached (PA) bacterial

community exhibited detectable shifts when exposed to different temperature regimes which were designed to simulate the warming in the Baltic Sea due to climate change during winter and spring (Houghton et al. 2001). The structure of the non-attached (NA) bacteria assemblage, however, did only marginally respond to rising temperature. Regarding the notion that inorganic nutrients do affect the bacterial community composition, and considering the combined influence of temperatures and nutrients on bacterial growth, the question arises whether these two factors also show a coupled effect on the structure of the bacterial assemblage. Considering the fact that different oceanic regions differ with regard to limiting primary nutrients and that nutrient conditions often tend to vary seasonally (Sala et al. 2002, Pinhassi et al. 2006), the effect of the ocean warming on bacterial communities and the carbon cycle may only be understood by considering both temperature and nutrient conditions.

We approached this question by setting up an indoor bottle experiment designed to stimulate an algal bloom with a planktonic community consisting of phyto- and bacterioplankton. Exposing the bottles to a temperature gradient as well as different nutrient conditions, we aimed to find out whether different limiting nutrient concentrations affected possible temperature-associated shifts in the bacterial community composition (BCC). The molecular techniques CARD-FISH, DGGE-fingerprinting and sequencing of excised bands were applied to screen the bacterial assemblage for structural changes on the group and phylogenetic level. To be able to match the results from our community analysis with the respective bacterial activity, bacterial production was assessed using leucine incorporation.

Materials and Methods

Experimental design and sampling

Bottle experiments were set up to stimulate an algal bloom under different temperature and nutrient conditions in climate chambers. Temperatures were set at 4, 8 and 12°C, and three replicates for each nutrient regime (N:P=5, N:P=30) were installed in each climatic chamber. Water from 10 m depth was collected from Boknis Eck close to the mouth of Kiel Fjord and allowed to age in the dark for several weeks to remove as much residual organic carbon as possible. Subsequently, 24 L of water were filtered over GF/F filters (Whatman) and 0.45 µm cellulose nitrate filters (Sartorius) to remove large particles as well as heterotrophic nanoflagellates, and finally filled into transparent Nalgene bottles (Nalgene, Rochester, USA). The remaining bacterial population was used as inoculum for the experiment. From a pure axenic culture of *Skeletonema costatum* (strain CCMP 1332) in exponential growth, 300 mL were added to a final concentration of ~700 cells mL^{-1}. Nitrogen and phosphorus concentrations were adjusted to the above mentioned ratios and silicate was added to ensure growth of the diatom *Skeletonema costatum* (final concentrations see Tab. 1). All bottles were exposed to the same light regime of a day-night-cycle of 12:12 hours. Light increased for six hours from ~155 to ~388 µmol photons m^{-2} s^{-1} and was reduced again down to ~155 µmol photons m^{-2} s^{-1} during the following six hours. Before each sampling, water was mixed by consecutively applying a magnetic stirrer in each bottle. Since sampled water was not replaced in order to avoid disturbance of the nutrient conditions and dilution of the initial water body, sampling volume and frequency was limited as much as possible. This resulted in 14 samplings at the warmest and 15 at the two colder temperatures. At each sampling, 1.5 L of water were decanted into clean glass bottles from which samples for the parameters described below were taken. Including the water sampled for chl *a* measurements (see below), total volumes of 21.2 L (12°C) and 22.72 (8°C, 12°C) were taken from the original experimental water body.

Tab. 1: Average final nutrient concentrations [µmol] after nutrient addition at the start of the experiment in 1) original aged sea water, 2) high N-concentrations and 3) low N-concentrations

	nitrate	ammonium	phosphate	silicate
sea water (aged)	5.8	1.9	0.3	5.9
high N-concentrations	23.8 ± 1.7	1.7 ± 0.1	0.87 ± 0.06	43.8 ± 0.4
low N-concentrations	5.4 ± 0.7	1.7 ± 0.1	0.87 ± 0.06	43.8 ± 0.4

Nutrients and chlorophyll *a* concentration

For the assessment of ammonium (Holmes et al. 1999), unfiltered water samples were used. Nitrate, nitrite and phosphate were determined after Hansen and Koroleff (Hansen & Koroleff 1999) after water samples had been prefiltrated through 0.65 cellulose acetate filters. All measurements were done on the day of sampling.

Water samples of 5-10 mL were taken from each treatment and dark adapted for 10 minutes for a rapid analysis of chl *a* dynamics by measuring the relative fluorescence with a PhytoPAM device (Walz, Germany), equipped with an optical unit ED-101US/MP. This was necessary for a realtime survey of the algal development in order to determine crucial stages of the algal bloom at which sampling for bacterial parameters was of paramount importance.

Enumeration of bacteria

Non-attached (NA) Bacteria were counted with a flow cytometer. 4 ml of a sample were incubated with 400 µl paraformaldehyde (1 % final concentration)/ glutaraldehyde (0.05 % final concentration) in the dark for one hour at 5°C. After fixation, the samples were frozen in liquid nitrogen and stored at -80°C. Heterotrophic bacteria were stained with SYBR Green (2.5 µM final concentration, Molecular Probes) for at least 30 minutes in the dark. Cells were counted using a Becton&Dickinson FACScalibur equipped with a laser emitting at 488 nm at a constant flow rate (35 µl / min). Yellow-green latex beads (0.5 µm, Polysciences) were used as an internal standard. Bacteria were detected by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1).

For the enumeration of particle-attached (PA) bacteria, 100 mL from each water sample were fixed with glutardialdehyde (EM grade, 1% final concentration) and stored at 4°C until further processing. Numbers of particle-attached bacteria were assessed after filtration of 6 mL of pretreated water over black 3.0 µm polycarbonate filters. Cells were stained with DAPI (4′-6-diamidino-2-phenylindole) and counted under an epifluorescence microscope (Axioskop plus, Zeiss, Germany) at 1000x magnification. In a minimum of 15 view fields, between 600 and 850 bacterial cells were enumerated on average, a guideline that could not always be met at early stages of the bloom when the occurrence of particles was very low.

Bacterial production

Bacterial secondary production measurements were conducted following the slightly altered protocol of Simon & Azam (1989); water was not pre-filtrated, the extraction was done with ice cold TCA without the washing step and filters were added directly to the scintillation solution. Three aliquots (2 replicates and one blank) of 10 ml of water were each incubated with 50µl of a 0.1µCi/10µl ^{3}H-leucine solution (specific activity: 77µCi/nmol) plus 50µl of a 2nmol/100µl unmarked leucine solution, resulting in a saturating concentration of 106,49 nmol/L of leucine in the sample. All samples were incubated in the respective climate chambers at in situ temperature in the dark for 1.5-3 hours. Incubation was terminated by the addition of formaldehyde (1% v/v) and cells filtered onto 0.2µm polycarbonate filters. The filters were subsequently rinsed with ice cold 5% TCA (trichloro acetic acid) solution, before being radio-assayed in 4ml of scintillation cocktail (Lumagel Plus) using a Packard Tricarb counter.

Filtration, isolation and PCR amplification of nucleic acids

Immediately after sampling, 500 to 600 ml water were filtrated onto two differently pore-sized polycarbonate filters (diameter 47mm, Osmonics, MN, USA). To distinguish between particle-attached and non-attached bacterial assemblages, seawater was fractionated by filtration onto 3.0 µm and subsequently onto 0.2 µm polycarbonate filters. The filters were shock-frozen in liquid nitrogen and stored at -20°C until further processing. Isolation of nucleic acids was performed by a pH-sensitive, phenol-chloroform extraction to obtain DNA as well as RNA (Süß et al. 2006). However, only RNA samples were processed further by first being transcribed into cDNA by an RT-step, using iScript TM Select and the included protocol (Bio-Rad, Munich, Germany ; primer 1492r, (Lane 1991)), and subsequently used as template for DGGE-PCR.

PCR was performed with a Bio-Rad my-cycler (Bio-Rad, Munich, Germany), using primer pairs specific for *Bacteria* 358fGC (Muyzer et al. 1993), and 907rM (Muyzer & Smalla 1998). Amplification of *Bacteria*-specific 16S rcDNA gene fragments was performed using a touchdown program with decreasing annealing temperatures from 65 to 55°C (2 cycles per step) and subsequent 16 standard cycles. 1 µl (1 to10 ng) of extracted DNA served as template. For quantification of the PCR-products, 4 µl of the amplification products, along with a quantitative ladder (1kB, Eppendorf), were analyzed by electrophoresis in 1,2% (w/v)

agarose gels. The gels were stained with ethidium bromide and visualized under UV-light (Gel Doc XR, Bio-Rad, Munich Germany).

DGGE and phylogenetic affiliation

DGGE was performed with the Ingeny Phor-U system (Ingeny, Goes, Netherlands), using the protocol described by Brinkhoff & Muyzer (Brinkhoff & Muyzer 1997), except for the applied denaturating gradient of the gel which was 20-70% . After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes, Inc.) and documented using the Gel Doc XR system (Bio-Rad, Munich, Germany). Prominent bands were excised and transferred to sterile Eppendorf caps using an ethanol-sterilized scalpel. Fifty µl of water (molecular grade, Eppendorf, Germany) were added, samples were incubated at 4°C overnight for resuspension of cDNA and finally stored at –20°C.

The banding patterns and relative quantity of bands were analyzed using the software Gel Compar II, version 2.5 (Applied Maths, Kortrijk, Belgium). Cluster analysis in order to compare banding patterns was done band based on presence-absence data with the binary Dice coefficient for similarity measurement of bands and the dendogram type UPGMA (**U**nweighted **P**air **g**roup **M**ethod using **A**rithmetic averages). The normalization to the same standard of both the NA and PA gels allowed a joint cluster analysis of both assemblages.

Excised bands (nomenclature AS07-NA/PAxy_z for **A**QUA**S**HIFT 20**07**, **n**on-**a**ttached or **p**article-**a**ttached) were sequenced by JenaGene (Jena, Germany) using the primer pair 358f and 907rM. For all sequences, at least 350 bp were analysed with the software SeqMan (DNASTAR, Wisconsin USA). Phylogenetic affiliation was determined by using the BLAST function of the NCBI server (http://www.ncbi.nlm.nih.gov) to perform comparison with the data available in GenBank, where the sequences obtained in this study are filed under the accession numbers EU068005 – EU068025.

CARD-FISH analysis

After fixation with glutardialdehyde (4% w/v) for one hour, 40 ml water were filtrated onto 3.0 µm and subsequently onto 0.2 µm polycarbonate filters (diameter 47mm, Osmonics, MN, USA) to distinguish between particle-attached and non-attached bacterial assemblages. Until further processing, filters were stored in the dark at -80°C. CARD (**ca**talysed **r**eporter **d**eposition)-FISH was performed according to Sekar and co-workers (2003) with the following modifications: After the agarose treatment, filter snips were dried with the surface upward on object slides and covered with 96% ethanol before being peeled off again. Permeabilisation, hybridisation and the actual CARD (Grote et al. 2007) were performed in 1.5 ml reaction tubes (Eppendorf), containing six filter snips at most per step. For characterization of the bacterial community, the following horseradish peroxidase probes (Biomers.net, Ulm, Germany) were applied: EUB 338-I-III (targeting most *Eubacteria*, (Amann et al. 1990, Daims et al. 1999), ALF968 (targeting most α-*proteobacteria*, (Neef et al. 1999), BET (targeting most β-*proteobacteria*, Manz et al. 1992)**,** GAM42a (targeting most **γ-***proteobacteria*, Manz et al. 1992), CF319 (targeting many groups belonging to the *Bacteroidetes* group**,** Manz et al. 1996). The EUB antisense probe NON338 (Wallner et al. 1993) served as a negative control. After hybridization and CARD, filter snips were counterstained with Vectashield-mounting solution with DAPI (1 µg mL^{-1}). Enumeration of DAPI-positive cells (between 500 and 1.000) and hybridisation signals were counted in a minimum of 15 fields (1000x magnification), using epifluorescence microscopy (Axioskop, Zeiss, Germany).

Results

Nutrient, algal and bacterial dynamics

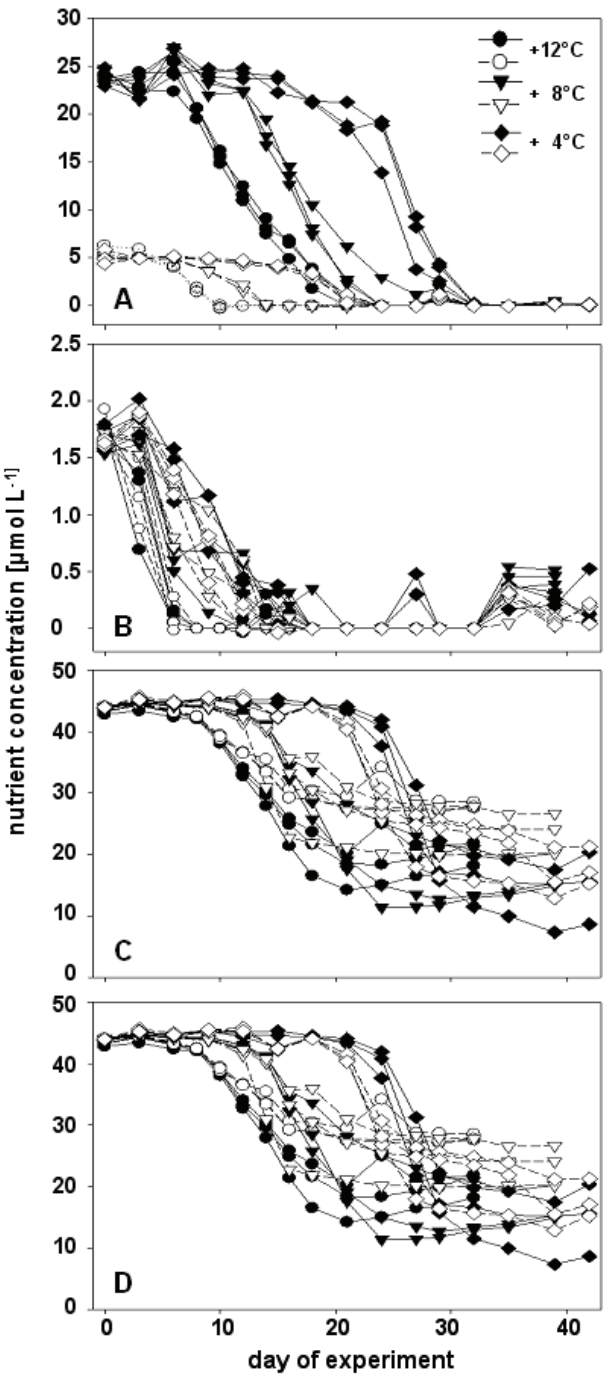


Fig.1: The dynamics of the nutrient concentrations during course of experiment. Nitrate (A); ammonium (B), silicate (C) and phosphate (D) concentrations are given for each temperature treatment and parallel; dark symbols representing high N, open symbols standing for low N concentrations.

In all temperature and nutrient treatments, measurements of chl *a* revealed the progression of distinct algal blooms. With the exception of the first bottle of the high-N treatment at +8°C, which showed a distinct diversion from the other two replicates, phytoplankton seemed to develop strikingly parallel in all three replicates of all treatments (Fig.2). However, phytoplankton growth and peak formation was more pronounced at higher N-concentrations at all temperatures. In the +12°C treatments, chl *a* measurements reached maximum values from 12.9 to 16.2 at high and from 3.9 to 4.3 at low N-concentrations. With maximum measurements ranging from 11.2 to 20.5 (high-N conditions) and from 3.8 to 6.5 (low-N conditions), as well as from 20.2 to 24.3 (high-N conditions) and from 4.3 to 5.6 (low-N conditions), a similar trend was obtained for temperatures +8°C and 4°C, respectively. Furthermore, the peak of the phytoplankton bloom occurred later with decreasing temperatures at both nutrient concentrations. However, this development was more pronounced in the high-N treatments, were maximum chl *a* concentrations were reached on

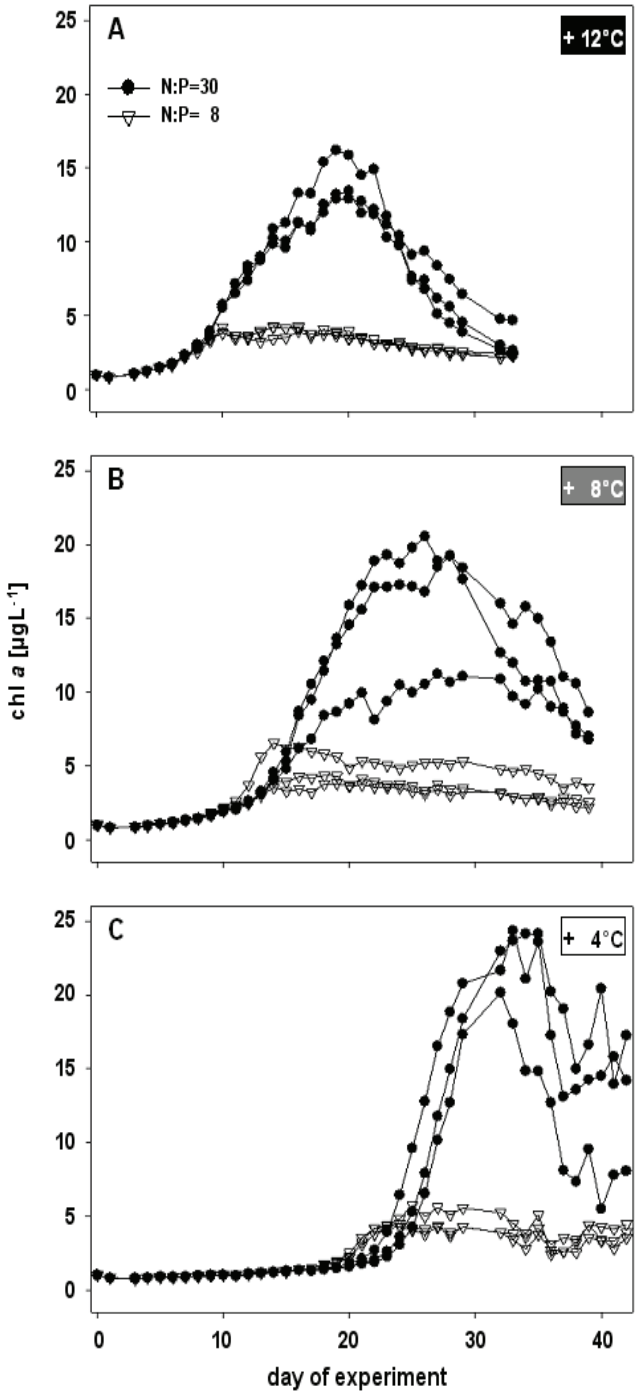


Fig.2: Chlorophyll *a* concentration for both parallels during course of experiment for all three temperature treatments +12°C (A), +8°C (B) and +4°C (C). Plotted are concentrations measured by PhytoPAM for all three replicates at high N (black) and low N concentrations (open symbols).

days 19-20 (+12°C), 26-28 (+8°C) and 31-32 (+4°C; Fig. 1 A-C), displaying a time lag of almost one week between warmest and medium, and of five days between medium and coldest treatments. This trend is in accordance with the nutrient dynamics, which showed a temperature-associated decline of nitrate, ammonium, phosphorus and silicate concentrations at both nutrient treatments, with the decrease of ammonium revealing the weakest link to temperature.

Additionally, our analysis revealed increasing chl *a* values with declining temperatures at high N concentrations (Fig.2), where concentrations of chl *a* revealed an average maximum of 14.2, 19.9 and 22.9 µg L^{-1} at 4, 8 and 12°C, respectively (Fig.2). Algal dynamics in the low N treatments, however, exhibited similar chl *a* concentrations at all temperatures (4.14, 4.9 and 4.9 µg L^{-1} at 4, 8 and 12°C).

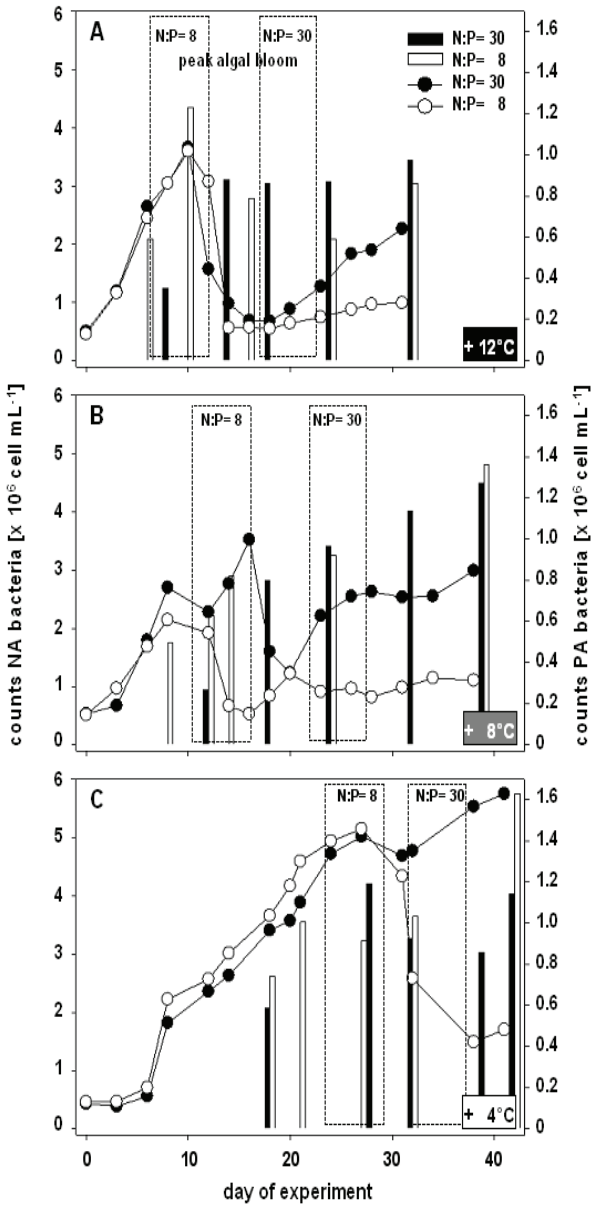


Fig.3: Counts for NA (curves) and PA (bars) bacterial numbers during the course of the experiment. Shown are the data for one representative replicate (exemplary for the general progress of quantitative bacterial development) for each temperature treatment [12°C (A), 8°C (B) and 4°C (C)] and N concentration. The time period of the phytoplankton peaks are given as framed areas for each nutrient treatment.

In the high N treatments, numbers of non-attached bacteria showed a peak during the exponential phase of the phytoplankton bloom at all temperatures (Fig.3 A-C). At low N concentrations, the bacterial and algal peak coincided only in the 12°C and the 4°C treatments. At 8°C, the bacterial maximum preceded that of phytoplankton by several days (Fig.3 B). As with chl *a*, highest NA bacterial numbers were found at coldest temperatures where they reached 5.5 x 10^6 cells mL^{-1} at high and low N concentrations, albeit at different points of time (Fig.3 C). At 8 and 12°C, NA bacterial numbers declined sharply after their first peak at both N concentrations and increased again during the late stage of the experiment at high-N conditions, whereas low-N NA-bacterial numbers recovered only fractionally. In the coldest treatments however, high-N counts of non-attached bacteria increased steadily over time, reaching their maximum shortly before the algal maximum (Fig.3 C). Low-N NA-bacterial numbers did show the same breakdown as at warmer temperatures, but did not recover again during the sampling period.

In all temperature and nutrient treatments, numbers of PA bacteria increased after initial sampling at the beginning of the phytoplankton bloom and remained high during the progress of the algal development, though some fluctuations appeared during the late state of the phytoplankton bloom in the counts for low N-concentrations at 12°C and the high-N treatment at 4°C (Fig.3 A and C). PA bacterial counts were highest at 8°C and 4°C in both high and low-N treatments, reaching maximum values of 1.27 and 1.36 x 10^6 cells mL^{-1}, and

1.19 and 1.63 x 10^6 cells mL^{-1}, respectively. For both nutrient approaches, PA bacterial numbers were lowest over all at highest temperatures with maxima of 0.97 x 10^6 cells mL^{-1} in high-N and 1.23 x 10^6 cells mL^{-1} in low-N treatments.

Bacterial production

Within the low-N treatments, bacterial production showed an initial increase in Leu-incorporation up to 2.3 pM/h at warm temperatures, and levelled out at uptake rates between 0.3 as minimum and 2.2 pM/h as maximum values at all temperatures (Fig. 4 B). In none of the low-N treatments, did the maximum of bacteria production coincide with the peaks in the algal development .

In contrast, Leu-incorporation at high N concentrations appeared to be linked with algal dynamics, reaching maximum values at the respective phytoplankton peaks, with only one treatment at +8°C increasing even further after maximum chl *a* concentrations were reached (Fig.4 A). Furthermore, bacterial production at high N-concentrations showed similar incorporation rates at warmest and medium temperatures, but were markedly lower in the coldest treatment, where uptake rates of leucine resembled those of the low-N treatments.

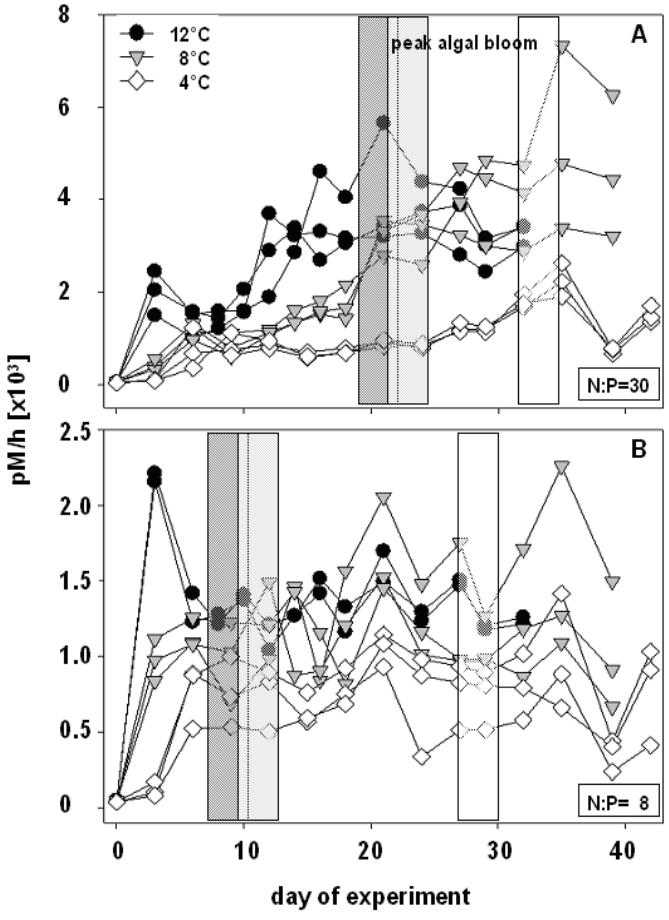


Fig.4: Leucine incorporation for high (A) and low (B) N concentrations. Presented are incorporation rates for all three parallels at the temperatures 12°C (black), 8°C (grey) and 4°C (white). Framed areas represent the peak of the phytoplankton blooms at 12°C (dark grey), 8°C (light grey) and 4°C (white).

Bacterial community composition

Group specific structure

A complete CARD-FISH analysis of all selected points during the algal bloom from all temperature treatments and both nutrient concentrations was not possible. In both fractions, some samples could not be successfully hybridized, which was especially true for the probes targeting α-*Proteobacteria* and the *Bacteroidetes* cluster in the non-attached fraction of the bacterial community (Fig.5 A, E, D and H). From the results that were obtained from the remaining samples, α-*Proteobacteria* still seemed to be the most abundant bacterial group among the tested probes at all analysed temperatures and both nutrient treatments. Counts of hybridisation signals reached up to 38.2% DAPI (high-N treatment) and 43% (low-N treatment) at +12°C and 41.9% DAPI (high-N treatment) and 37.4% (low-N treatment) at +8°C. For the +4°C treatment, no hybridisation signals could be obtained. Members of the group β-*Proteobacteria* were least abundant at all temperatures and both nutrient concentrations (Fig.5 B and F). Still, the β-*Proteobacteria* counts from t_1 obtained at coldest temperatures and high N concentrations were notably higher than at +8 and +12°C and at low-N conditions. Percentages of γ-*Proteobacteria* ranged between 12.5 % DAPI (+4°C) and 0.7% (+12°C) at high, and from 18.3% DAPI (+4°C) and 1.7% (+8°C) at low N concentrations (Fig. 5 C and G). Members of the group *Bacteroidetes* were only marginally detectable in high-N treatments (one point of time for each temperature treatment), where they increased in proportion with declining temperatures at both N. A similar trend, albeit not as distinct, was found at low-N concentrations. An over-all analysis of any dynamics over the course of the algal bloom was not possible due the insufficient amount of data.

In the PA fraction, hybridisation was more successful; only a few samples were not suitable for analysis (Fig.6 A, E and B, F). In contrast to the NA fraction, α-*Proteobacteria* were the least abundant group in both nutrient treatments, along with the β-*Proteobacteria*, reaching similar proportions at all temperatures with maximum values of 5.3% DAPI (+8°C) at high N and 8.3 (+8°C) at low N conditions. For both N-concentrations, γ-*Proteobacteria* were most abundant in the warmest treatments, though this trend was more pronounced at high-N concentrations. *Bacteroidetes* revealed highest percentages in both nutrient treatments of the PA fraction, reaching maximum proportions of 52.9 % (+4°C, high-N conditions) and 76.5% DAPI (4°C, low-N conditions) (Fig.6, D and H). Especially for the proportions of γ-*Proteobacteria* and *Bacteroidetes*, dynamic developments could be detected over the algal bloom, which showed differing trends between temperature as well as nutrient treatments.

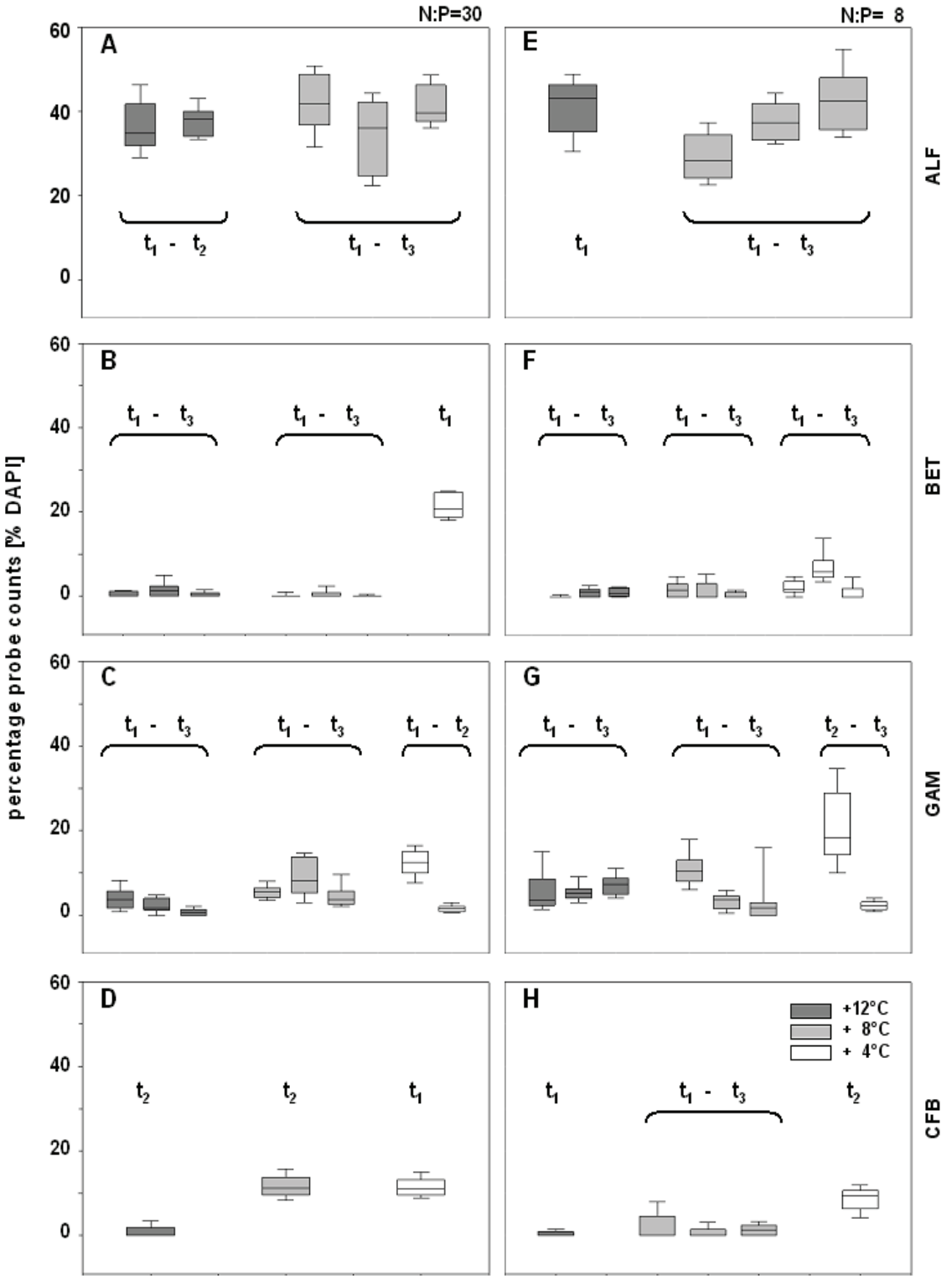


Fig.5: CARD-FISH counts of bacterial groups α- (ALF), β- (BET), γ-*Proteobacteria* (GAM) and *Bacteroidetes* (CFB) for the NA bacterial community. Shown are percentages for high (A-D) and low N conditions (E-H). Plotted is one selected parallel at three cardinal points of the algal bloom (t_1 = exponential phase, t_2 = peak, t_3 = degradation phase). All values of single counting grids were included in calculation to display median values and counting variability. Box-Whisker-plots show the $25^{th}/75^{th}$ percentile (box), the mean (solid line) and the $5^{th}/95^{th}$ percentile (whiskers).

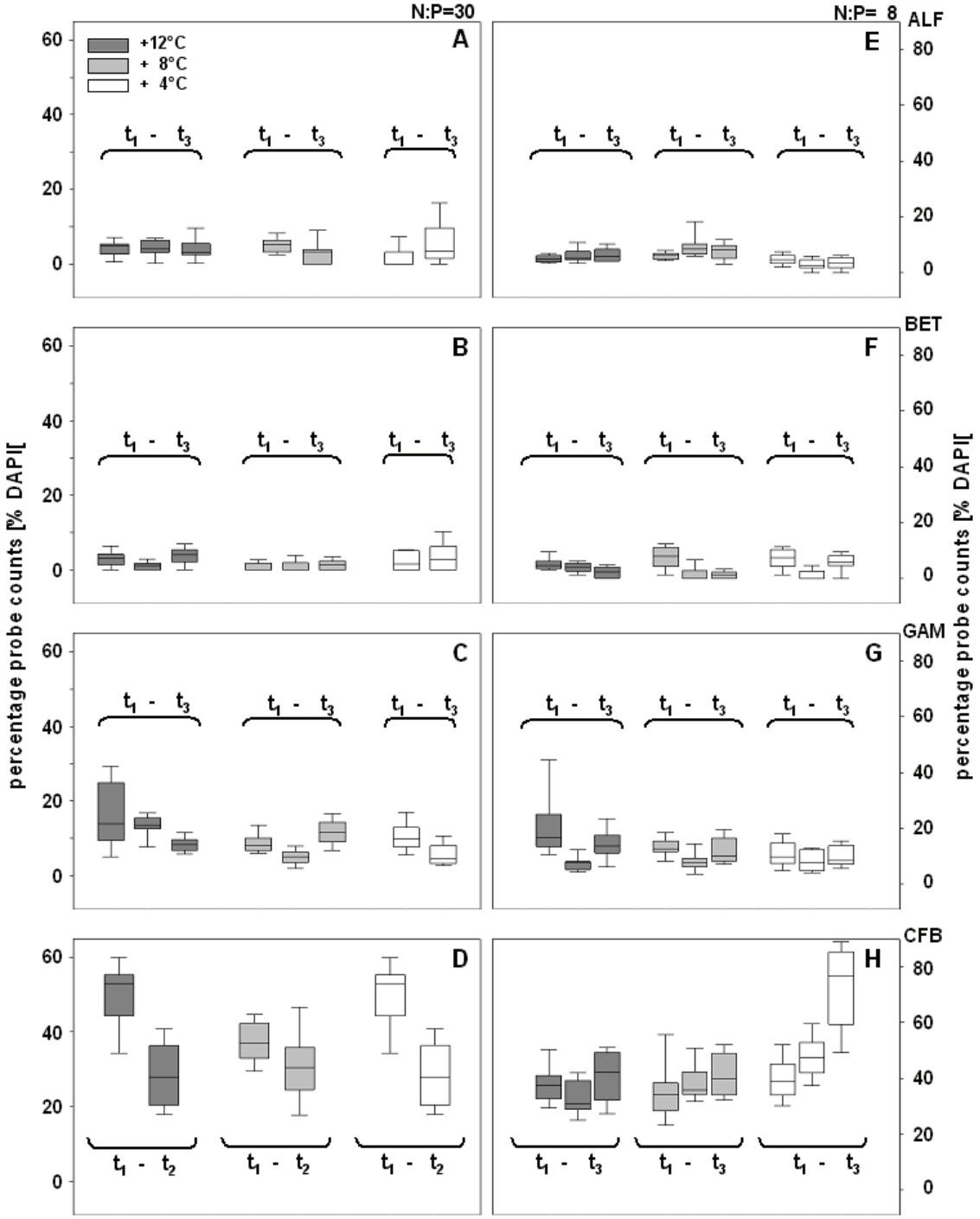


Fig.6: CARD-FISH counts of bacterial groups α- (ALF), β- (BET), γ-*Proteobacteria* (GAM) and *Bacteroidetes* (CFB) for the PA bacterial community. Shown are percentages for high (A-D) and low N conditions (E-H). Diagrammed is one selected parallel at three cardinal points of the algal bloom (t_1 = exponential phase, t_2 = peak, t_3 = degradation phase). All values of single counting grids were included in calculation to display median values and counting variability. Box-Whisker-plots show the $25^{th}/75^{th}$ percentile (box), the mean (solid line) and the $5^{th}/95^{th}$ percentile (whiskers).

The *Bacteroidetes* cluster showed the most distinct dynamics within the PA fraction, with a clear trend of decreasing counts from t_1 to t_2 at all temperatures in the high-N treatments. At the extreme temperatures, proportions of *Bacteroidetes* declined from 59.4 to 29.2 % DAPI at +12°C and from 52.9 to 27.9% DAPI at 4°C; the +8°C treatment exhibited a drop from 36.9 to 30.4 % DAPI. At low-N conditions, the decrease in *Bacteroidetes* counts was within the same range at 12°C, but the two colder treatments showed growing proportions of with the progress of the algal bloom. This development was clearest at +4°C with an increase from initial percentages of 38.8% DAPI to 76.5% DAPI at t_3.

Genetic fingerprints, cluster analysis and phylogenetic affiliation

Five points during the overall algal development were selected to take samples for the analysis of the bacterial community on the species level. We chose bloom onset, middle of the exponential phase, peak of bloom, middle and end of degradation phase as cardinal points for a bacterial genetic fingerprinting of the NA and PA fraction from each temperature and nutrient concentration. The mainly parallel development of algal and bacterial dynamics allowed us to choose one of the three nutrient parallels at each temperature for DGGE analysis. Where the genetic fingerprints show less than five lanes for a nutrient and temperature specific treatment (Fig.7 A, B and Fig.8 A, B), PCR amplification of the missing samples was not possible.

In the bacterial NA fraction at high-N conditions, the number of bands obtained by DGGE varied between 7 and 10 at all temperatures (Fig.7 A). At low-N concentrations, the number of NA ribotypes was higher in the +12°C and +4°C treatments than at +8°C, ranging between 9 and 14 bands and decreasing from the middle towards the end of the algal bloom at both temperatures (Fig.7 B). In both nutrient treatments, there were several bands prevailing at all temperatures (AS07-NA2_0, AS07-NA6_0, AS07-NA9_0 and AS07-NA15_0 at high-N conditions as well as AS07-NA4_1, AS07-NA5_1 and AS07-NA6_1 at low N concentrations), whereas at high-N conditions, AS07-NA3a_0, AS07-NA10_0 and AS07-NA11_0, and the low-N band AS07-NA8_1 were only present at one particular temperature. AS07-NA8_1 could successfully be identified by sequencing and was found to belong to the *Bacteroidetes* clade (Tab.1) with the closest cultivated relative being *Fluviicola taffensis* (Tab.1, 92% sim.), a freshwater bacterium. Furthermore, changes in band intensity were observed over the course of the algal bloom at all temperatures and nutrient concentrations. In the high N-treatments, for instance, the ribotype AS07-NA6_0 and its equivalents at the same gel position at other temperatures increased in intensity, whereas AS07-NA2_0 grew less pronounced over the bloom (Fig.7 A). At low N-concentrations, the bands AS07-NA1_1 and -NA2_1 were only present before the algal peak and only at the extreme temperatures; AS-NA5_1 increased in intensity with the progressing phytoplankton bloom at all temperatures.

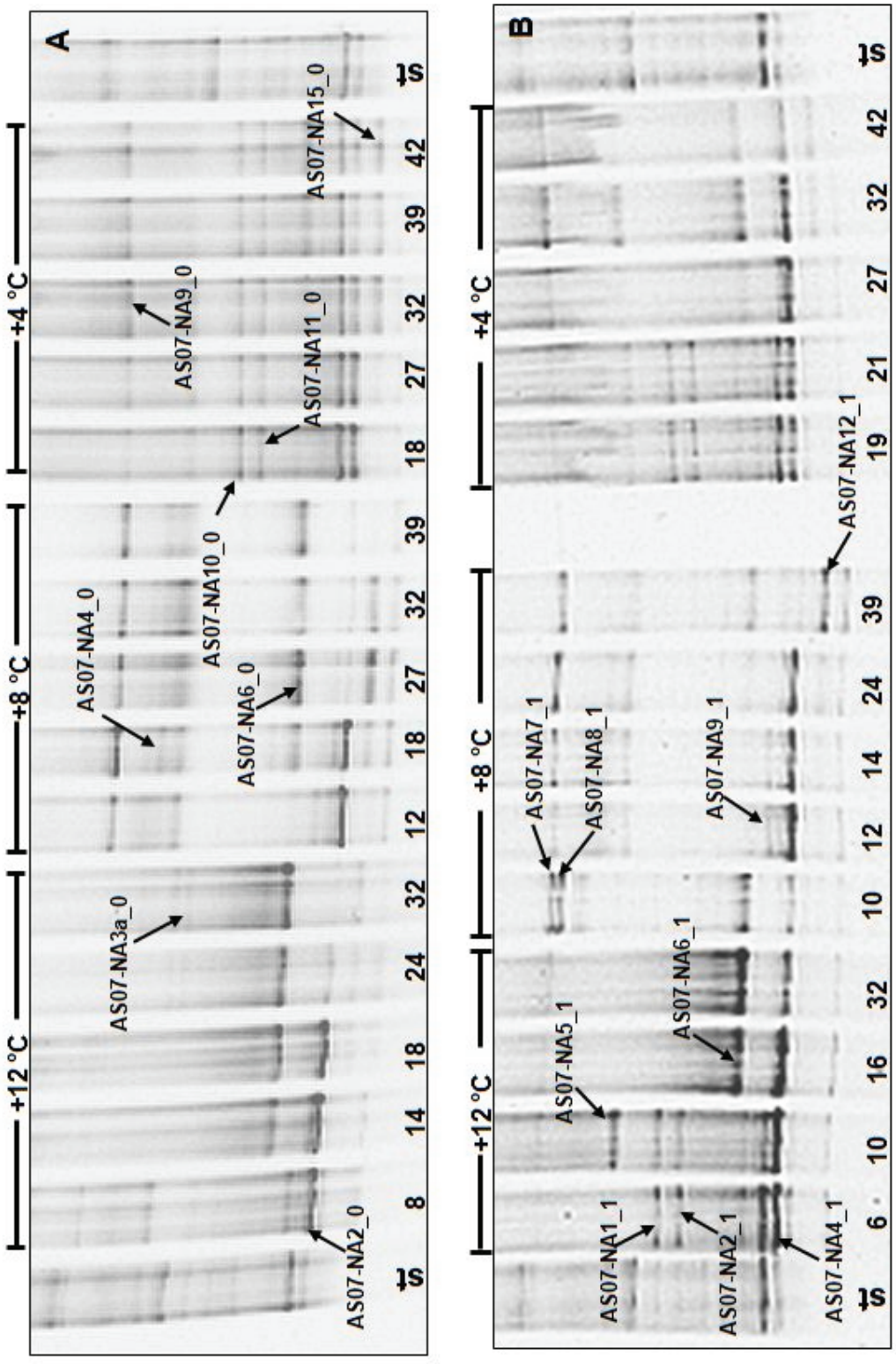


Fig.7: DGGE fingerprints of 16S rRNA based cDNA (eubacterial primers 358f GC and 907rM) for one parallel of the non-attached [NA] bacterial fraction at high (A) and low (B) N concentrations for several points of time over phytoplankton bloom at three temperature treatments (+12, +8 and +4°C). Numbers show day of sampling, arrows mark excised and sequenced bands (see Tab.1 for band affiliations).

Table 1: Phylogenetic affiliation, band Identification (band ID) of excised bands for the bacterial non-attached [NA] and particle-attached [PA] fraction at all temperatures and low as well as high N concentrations. Further given are closest (cultivated) relative with accession number, sequence similarity, taxon and habitat.

Band ID	closest cultivated relative (acc. no.)	similarity [%]	taxon	habitat/environmental features (closest relative)
AS06-PA 1_2	uncult. *Flavobacterium* clone	100%	*Bacteroidetes*	marine (North Sea)
AS06-PA 2_2	*Microscilla furvescens*.; M58792	93%	*Bacteroidetes*	marine
AS06-PA 4_2	uncult. *Bacteroidetes* bacterium; AM851001	87%	*Bacteroidetes*	marine
AS06-PA13_2	*Flexibacter aggregans*; M78591	92%	*Bacteroidetes*	marine, glob. distr.
AS06-PA 1_4	*Microscilla furvescens*.; M58792	93%	*Bacteroidetes*	marine
AS06-PA 8_4	*Skeletonema costatum*; X82154	99%	eukayote	marine, temperate waters
AS06-NA 2_0	uncultured *Hydrogenophaga* sp.; DQ421774	98%	β-*Proteobact*	coastal seawater
AS06-NA 6_0	*Microscilla furvescens*.; M58792	94%	*Bacteroidetes*	marine
AS06-NA 9_0	*Loktanella vestfoldensis*; AJ582227	99%	α-*Proteobact.*	marine, pelagic/attached
AS06-NA15_0	*Flexibacter aggregans*; M78591	92%	*Bacteroidetes*	marine, glob. distr.
AS06-NA 4_1	*Skeletonema costatum*; X82154	99%	eukayote	marine, temperate waters
AS06-NA 5_1	*Owenweeksia hongkongensis*; AB125062	88%	*Bacteroidetes*	marine
AS06-NA 7_1	uncult. *Bacteroidetes* bacterium; AM851001	93%	*Bacteroidetes*	marine
AS06-NA 8_1	*Fluviicola taffensis*; AF493694	92%	*Bacteroidetes*	fresh water
AS06-NA 12_1	*Microscilla furvescens*.; M58792	94%	*Bacteroidetes*	marine

Cluster analysis for the NA fraction revealed different patterns for both nutrient treatments (Fig.8 A and B). At low-N conditions, no temperature linked grouping was evident; only the samples from +8°C formed one self-contained cluster, whereas coldest and warmest communities did not separate from each other. At high N concentrations, however, two main clusters formed, within which the bacterial community from the coldest temperatures was markedly different from those at the two warmer treatments. The only samples not sorting into these clusters were taken at the two initial points of time from the +12°C treatment.

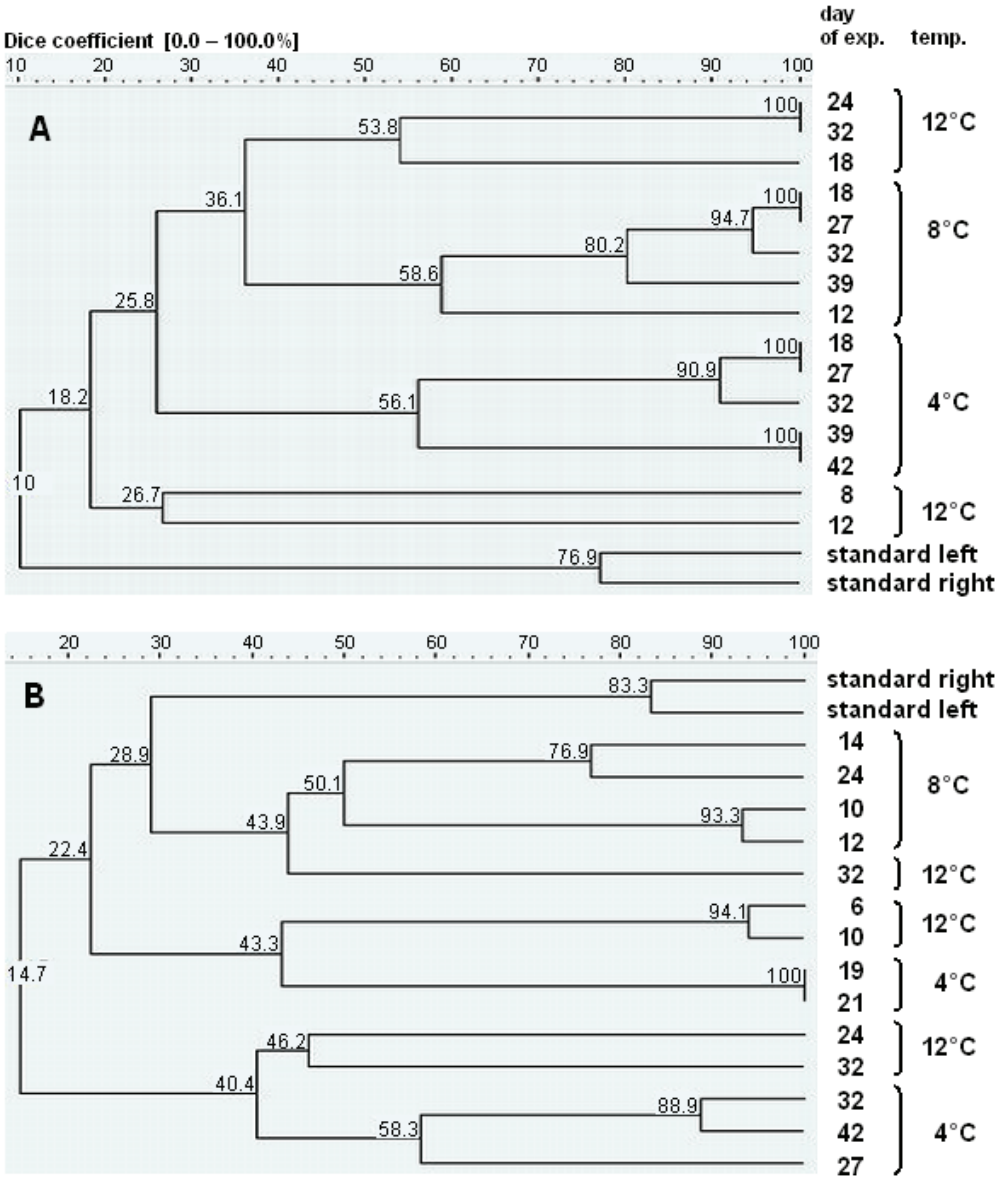


Fig.8: Curve based cluster analysis (UPGMA = unpaired group method of analysis) of DGGE fingerprint banding patterns (see Fig.7) of the non-attached [NA] bacterial fraction at high (A) and low (B) N concentrations and three temperature treatments (+12, +8 and +4°C). Numbers in branches stand for calculated cophonetic correlations of each branching.

The PA bacterial community exhibited a definite increase in number of bands in the high-N treatments at coldest temperatures, a trend that was not detectable at low N conditions (Fig.9 A and B).

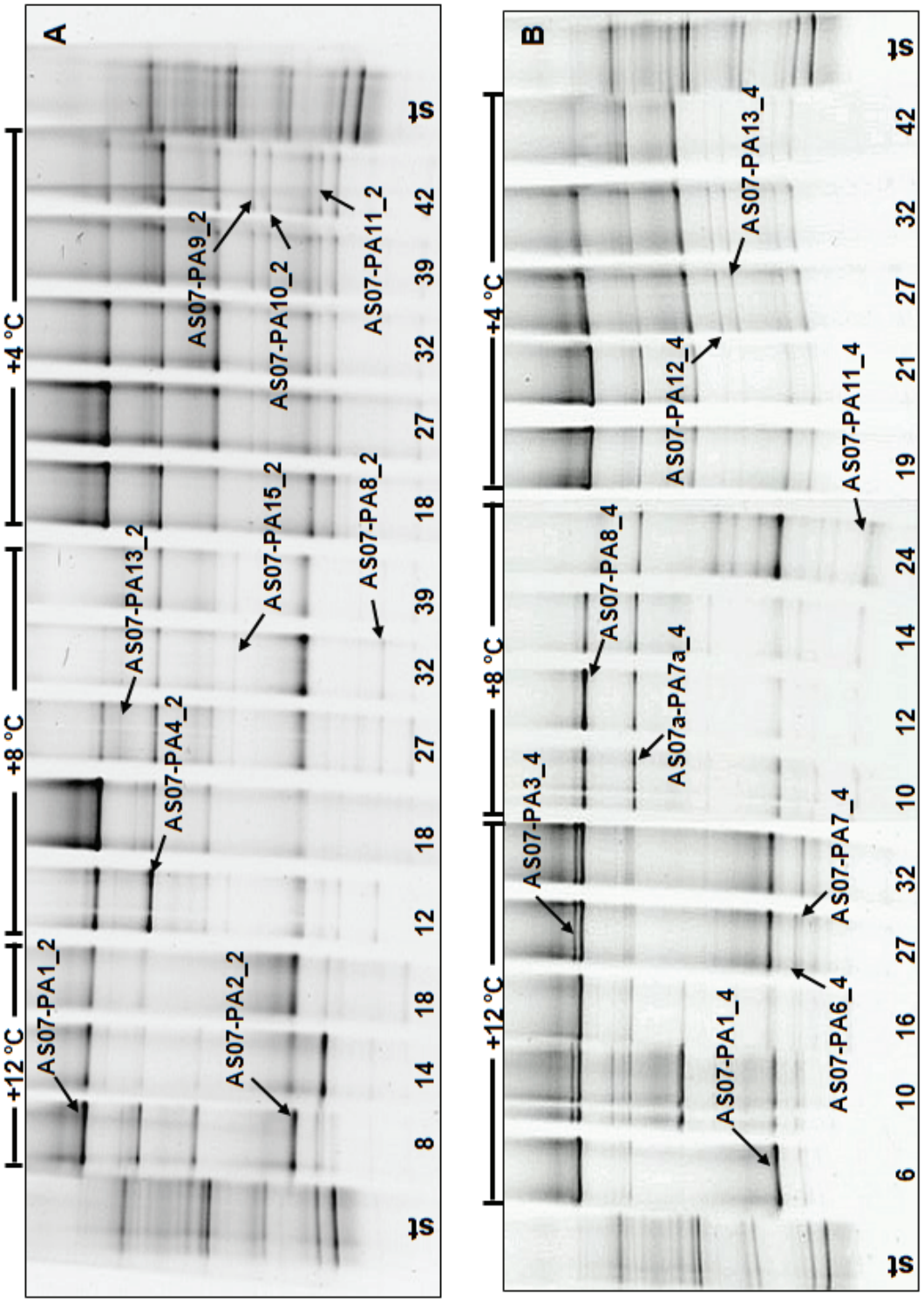


Fig.9: DGGE fingerprints of 16S rRNA based cDNA (eubacterial primers 358f GC and 907rM) for the particle-attached [PA] bacterial fraction at high (A) and low (B) N concentrations for several points of time over phytoplankton bloom at three temperature treatments (+12, +8 and +4°C). Numbers show day of sampling, arrows mark excised and sequenced bands (see Tab.1 for band affiliations).

As in the NA fraction, some detected ribotypes were constantly present, whereas others only appeared in particular treatments. At high N concentrations, the bands AS07-PA1_2 and AS07-PA4_2 were found at all three temperatures; AS07-PA8_2, AS07-PA13_2 and AS07-PA15_2 were detected only at +8°C, AS07-PA9_2 AS010-PA1_2 and AS07-PA11_2 were solely present at coldest temperatures (Fig.9 A). From the temperature-specific PA ribotypes in this nutrient treatment, one (AS07-PA13_2) could be successfully identified by sequencing and was affiliated with the *Bacteroidetes* phylum (Tab.1). Its closest cultivated relative was *Flexibacter aggregans* subsp *catalaticus* (NCIMB 1418; Tab.1, sim. 92%), which is also referred to as *Microscilla aggregans.* In the Low-N treatments, the number of bands restricted to one particular temperature declined markedly; only NA-PA11_4 merely appeared at +8°C. On the other hand, AS07-PA1_4, AS07-PA3_4, AS07-PA6_4, AS07-PA7_4, AS07-PA7a_4, AS07-PA12_4 and AS07-PA13_4 were present at all temperatures at at least one point of time during the algal bloom (Fig.9 B). According to these results, the PA bacterial fraction at high-N conditions exhibited the most temperature-specific bands of all treatments, whereas it showed the most uniform genetic fingerprint with seven omnipresent ribotypes at low N concentrations. In contrast to the not-associated bacterial community, the PA fraction did not show the same pronounced and rather stable trends of in- or decreasing band intensities.

Cluster analysis of the PA bacterial community revealed three distinct temperature linked clusters at high-N conditions (Fig.10 A and B). As within the NA fraction, the samples not falling into these groups were from the initial stages of the algal bloom. At low N concentrations, clustering with regard to temperature was more pronounced than in the NA fraction, but showed weaker similarity values than those from the high-N treatments. Again, the samples not clustering temperature related were taken from the first stages of the phytoplankton development.

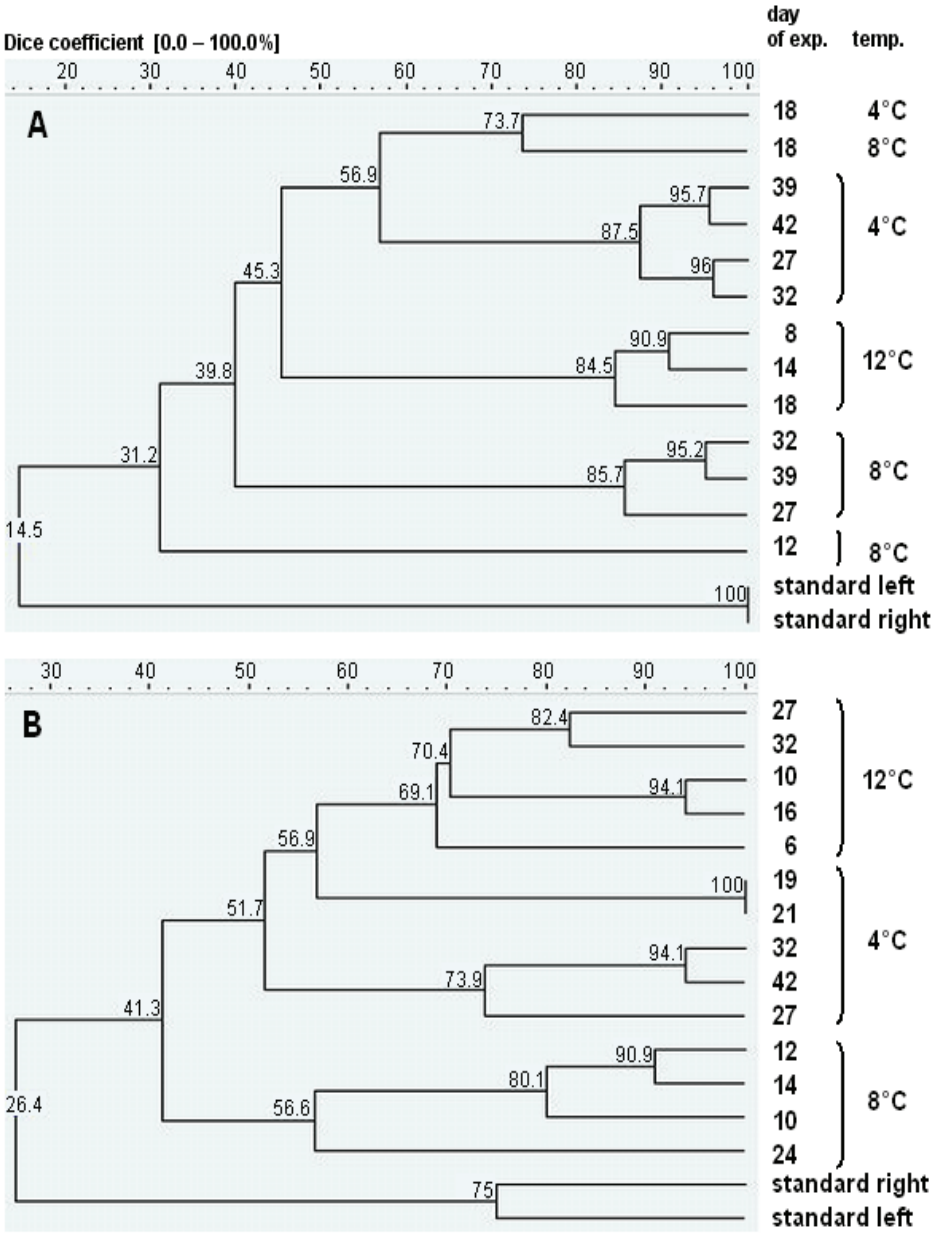


Fig.10: Curve based cluster analysis (UPGMA = unpaired group method of analysis) of DGGE fingerprint banding patterns (see Fig.7) of the particle-attached [PA] bacterial fraction at high (A) and low (B) N concentrations and three temperature treatments (+12, +8 and +4°C). Numbers in branches stand for calculated cophonetic correlations of each branch

Discussion

The experimental set-up was sufficient to generate blooms of the diatom *Skeletonema costatum* at three different temperatures and two nutrient regimes. The three parallel treatments for each nutrient concentration revealed similar developments for nutrient, phytoplankton and bacterial dynamics for the most part. This indicated that filtration and distribution of water, inoculation of phytoplankton and nutrient manipulation resulted in similar initial conditions and a resembling plankton standing stock for all treatments, and also suggests that were no treatment effects over the course of the experiment. Growth of *Skeletonema costatum* depended on the available N concentration, showing a more pronounced bloom at high-N compared to low-N conditions.

As in a previous experiment (Walther et al. in prep.), the bacterial community exhibited a composition already found in several other studies analysing bacterial assemblages in marine and estuarine habitats (DeLong et al. 1993, Bidle & Fletcher 1995, Acinas et al. 1999). Since the water used in this experiment was sampled from the brackish environment at Boknis Eck, the presence of one ribotype affiliated with the freshwater bacterium *Fluviicola taffensis* (Tab.1), though only found at +8°C, is consistent with the assumption of a brackish bacterial community that developed in this study. Furthermore, our CARD-FISH results showed that the PA fraction was dominated by members of the *Bacteroidetes* group. This finding is consistent with the presence of these bacteria on various kinds of particles (DeLong et al. 1993, Bidle & Azam 2001, Moeseneder et al. 2001), as well as their ability to degrade complex polymers (Reichenbach 1992, Kirchman 2002). In contrast, the groups α- and γ-*Proteobacteria* were most abundant in the non-attached fraction, which is consistent with the findings of other authors (Böckelmann et al. 2000, Bouvier & del Giorgio 2002, Simon & Tilzer 2002, Selje 2003, Castle, 2004). As already discussed in a previously conducted experiment (Walther et al. 2008, in prep), there were no sequences affiliated with γ-*Proteobacteria* among the excised bands from this study's fingerprints, though members of this subclass were detected in both bacterial fractions by CARD-FISH, especially in the PA bacterial community.

General algal-bacteria dynamics

Flowcytometer counts of non-attached bacterial cells and algal dynamics determined by chl a measurements showed similar trends for bacterial and phytoplankton development in the high-N treatments (Fig.2 and 3). Parallel increases in cell numbers and chl *a* concentrations indicate a coupling between phyto- and bacterioplankton.

At low-N concentrations, where chl *a* dynamics indicated a much weaker phytoplankton bloom, NA bacterial numbers at all temperatures remained within the same range during the first peak as at high-N conditions. At the two warmer treatments, the first bacterial peak formed over the first 18 days of the experiment; at 4°C, the first bacterial peak occurred later, but counts remained within the same range for both nutrient treatments. At all temperatures, DOC concentrations displayed similar dynamics during these first bacterial peaks (Wohlers et al. in prep., data not shown), irrespective of nutrient treatment and despite lower chl *a* concentrations at low N-conditions. These results point at a correspondence between the DOC concentration and the bacterial development. This notion is substantiated by the second increase of bacterial numbers during the last phase of the algal bloom which only occurred at high-N conditions. This development may be linked to the marked increase of DOC concentrations in the high-N treatments during the second stage of the experiment. On the other hand, DOC concentrations at low-N concentrations showed only a slight increase towards the end of the experiment which was restricted to warmer temperatures (Wohlers et al. in prep., data not shown). Again, the absence of a second peak of NA cell counts in the low-N treatments at all temperatures indicates a link between DOC concentrations and the NA bacterial development.

Even though water was filtrated previous to the start of the experiment in order to exclude grazers, control counts revealed the presence of heterotrophic nanoflagellates (HNF) in all treatments over the entire experiment (Breithaupt et al. in prep., data not shown). The sharp decline of NA bacterial numbers at both nutrient conditions in the warmer treatments could therefore be the result of HNF grazing, whereas an inhibited grazing activity at low temperatures could be the reason for the more or less steadily increasing high-N NA bacteria counts at 4°C. However, at no nutrient condition and at no temperature did HNF control counts suggest a link between HNF dynamics and the steep declines of the NA bacteria that occurred in all but the coldest treatments. It is noteworthy that similar dynamics of NA bacteria were documented for a previous experiment, where a striking decrease of highly active NA bacteria at higher temperatures could not be linked to HNF dynamics. Due to the

lack of ciliate counts during that experiment, ciliate grazing could not be excluded as a potential reason for the observed break down of highly active NA bacteria. For this study, however, microscopic analysis did not reveal the presence of any ciliates, most likely due to the filtration of the aged sea water prior to the experiment. This indicates that the distinct break down of NA bacterial numbers at all but the coldest temperatures was not a consequence of ciliate grazing.

The PA fraction of the bacterial assemblage is known for its role in colonising and degrading organic and therefore also algal particles (Hoppe et al. 1993, Middelboe et al. 1995, Becquevort et al. 1998), thus directly utilizing phytoplankton derived organic matter. The increase in numbers of PA bacteria with the progressing algal bloom and especially during the degradation phase at all temperatures and nutrient concentrations that we observed during our experiment was therefore expected (Fig.3 A-C). Moreover, our results suggest a functional, temperature-independent coupling between phytoplankton development and both bacterial fractions in both nutrient treatments.

The effect of temperature and nutrient regime on the bacterial production and community composition

Though DOC concentrations at both nutrient treatments remained within the same range during the first half of the experiment at all temperatures, leucine incorporation rates at warm temperatures reached maxima up to three times higher in high compared to low-N treatments (Fig.4 A and B). In contrast to bacterial dynamics, bacterial production apparently was not linked to DOC concentrations. A possible explanation for the nutrient-dependent difference between bacterial productions could be that the quality of dissolved organic carbon released by *Skeletonema costatum* was affected by the different nutrient regimes and N-concentrations, respectively. It has already been shown that growth of aquatic bacteria can be N-limited despite high DOC concentrations (Goldman & Dennett 2000). The low-N concentrations that were set in this study might therefore have induced such high DOC:N ratios of the algal-released dissolved organic matter that, as a consequence, bacterial activity was diminished at low-N conditions compared to high-N treatments. Qualitative analysis of DOM will shed some light on this assumption (Wohlers et al., in prep.)

In a study on the link between nutrient conditions, exudation of DOC by phytoplankton and bacterial growth, Obernosterer and Herndl (1995) showed that bacteria were not able to efficiently utilize the organic material released by P-limited algae during the exponential

stage, resulting in decreased bacterial growth yield. Since NA bacterial production in our high-N treatments was considerably higher than at low N concentrations, we were not able to confirm these findings. The different N:P ratios of 100 (Obernosterer and Herndl 1995) and 30 (present study) used in both studies which lead to different extents of P-limitation, might explain this discrepancy.

In contrast to the cold treatments, DOC-concentrations increased markedly and steadily at 8 and 12°C already after day 18. This points to a progressive release of dissolved organic carbon towards the peak and during the breakdown of the algal bloom (Wohlers et al. in prep., data not shown) at warmer temperatures and may be an explanation for the higher bacterial activity at 8 and 12°C compared to the cold treatment. Furthermore, the capability of the NA bacterial community itself for DOC uptake could have been reduced at cold temperatures (Pomeroy & Deibel 1986). Finally it as also possible that the P-limiting conditions at high-N concentrations resulted only at cold temperatures in the bacterial inability to utilize algal exudates (Obernosterer & Herndl 1995). The clearly diminished high-N bacterial production we observed at cold temperatures therefore indicates an interaction between ambient temperature and nutrient conditions, with warmer conditions mitigating the limiting effects of organic matter derived from algae under P-limiting conditions.

The apparent temperature effect on the bacterial activity at high-N conditions was mirrored in our genetic fingerprints for both bacterial fractions. While the NA communities from the low-N treatments did not reveal reliable temperature linked clustering, there was a markedly more distinct grouping with regard to temperature at high-N conditions. Though the PA communities at both nutrient conditions showed a generally clearer temperature related structuring than the NA fraction, higher similarity values in the cluster analysis hinted at a more pronounced temperature effect in the high-N treatments. For both bacterial fractions, the temperature linked clusters of the high-N treatments consisted of samples taken during the later stages of the algal bloom, whereas those sampled during the earlier phases tended to cluster separately. This indicates a growing temperature effect with the progress of the phytoplankton development. It is known that PA bacteria, and especially the *Bacteroidetes* group, are functionally linked to the degradation of phytoplankton blooms, to which they considerably contribute by enzymatically hydrolyzing complex organic compounds (Hoppe et al. 1993, Middelboe et al. 1995). Since these processes as well as bacterial substrate affinity are thought to be temperature dependent (Nedwell 1999), it is not surprising that we found a stronger temperature effect on the PA bacterial fraction during the degradation phase of the

algal bloom, as was already the case in a previous mesocosm experiment with similar design (Walther et al. in prep). Unfortunately, the inconstant CARD-FISH results were not sufficient for analysis in terms of temperature related shifts in group structure or with progress of the phytoplankton bloom. Particularly the missing counts for t_3 from the high-N treatments did not admit to affirm our theory of an intensified temperature effect at later algal bloom stages. Also, we are not able to explain why the occurrence of ribotype AS07-PA13_2 with the closets cultivated relative *Flexibacter aggregans* subsp. *catalaticus* (NCIMB 1418; Tab.1) was restricted to the +8°C treatment. The strain NCIMB 1418 was originally isolated from a cold habitat and is capable of growing at low temperatures, whereas it shows no nutritional versatility, but requires several nitrogen sources (Bowman et al. 2003), which might explain its absence at low-N conditions. Since *Flexibacter aggregans* was shown to be psychrotolerant (Bowman et al. 2003), however, we cannot clarify its absence in the +4°C treatments of the PA bacterial communities.

Reverting to the initial question, the presented results indicate that nutrient conditions do affect the extent of temperature-linked bacterial community structuring. While, irrespective of NA or PA fraction, the bacterial assemblages in our experiment generally tended to exhibit temperature linked structural differences during the late stages of the phytoplankton bloom, high N concentrations apparently enhanced the differences we found on the phylogenetic level. The findings of the present study therefore strongly suggest a combined effect of temperature and nutrient conditions on the bacterial community composition which should be taken into account in future analyses of the effect of rising ambient temperatures on marine heterotrophic bacteria.

Conclusions and outlook

In the study at hand, the influence of rising ambient tempertures on natural planktonic communities was analysed in temperature modulated mesocosms. The main focus was on the bacterioplankton assemblage, which was assessed for temperature linked shifts in the community structure on the group and species level by applying molecular biological methods. This work was carried through within the DFG proirity prgramm AQUASHIFT and contributes basic analyses on the possible influence of increasing water temperatures on the composition of marine bacterioplankton on which weiterführende more specific studies can be built/based upon.

Predicted future increases in temperature for the region of the Baltic Sea in the up-coming century range up to a rise of +6°C (BALTEX report 2006), with the focus of warming lying in the winter and early spring months. The hypothesis of different influences of these increasing temperatures on auto- and heterotrophic organisms is a central theory within the DFG priority project AQAUSHIFT. For the study at hand, it is the potential impact on the functional coupling between phyto- and bacterioplankton, especially possibly temperature-linked shifts within the bacterial communtiy structure which is of particular interest.

The experimental setting for the study at hand proved to be feasable. The results presented in **chapter 1** show that over the entire temperature gradient, distinct algal blooms developed from the overwintering Kiel Fjord phytoplankton stock in all mesocosms. Temperature as cardinal parameter was successfully held stable for the greatest part of the entire experiment in all climatic chambers; the dynamics of inorganic nutrients demonstrate that ressources were completely utilized by the present biota. Furthermore, it became clear that the planktonic community did respond to increasing temperatures. Especially the highly active fraction of the bacterial community showed a quantitative development which was markedly congruent with changing ambient temperatures. Yet, it was not possible to determine whether this was a direct temperature effect or mediated by top-down factors, which in turn might have been influenced by temperatures themselves. Therefore, the following chapters particularly focused on the analysis of the bacterial community.

The results of several studies have alraedy demonstrated the stimulating effect of rising temperatures on the activity of marine bacteria (Pomeroy & Wiebe 2001, Kirchman et al. 2005, López-Urrutia & Morána 2007). This parameter is of great importance for the degradation of organic material in the oceans and thus for the marine carbon cycle, which is why it is crucial to assess the the impact of future rising temperatures on the bacterial activity in the oceans; this question is also accounted for within AQUASHIFT. Still, the question

whether the bacterial community composition and therefore the qualitative utilization of organic material by bacteria is also affected by changing ambient temperatures has not found the same regard.

The analysis of the bacterial community composition, which is presented in **chapter 2**, therefore focused on probable temperature related shifts within the structure of the bacterial community. The statistic comparison of DGGE genetic fingerprints demonstrated that especially the particle-attached bacterial fraction, which is mainly responsible for the degradation of particulate organic matter, differed in community composition between the two analysed extreme temperatures. Also the CARD-FISH results exhibited different distributions of the tested bacterial groups, which seemed to be temperature-related. Sequencing of dominant DGGE bands showed that the bacterial assemblage that developed during the experiment consisted of species which were expected for the sampling site (Kiel Fjord). The observed temperature-linked shifts within the particle-attached bacterioplankton are therefore no result of the development of an artificial bacterial community, but could actually be the consequence of different ambient temperatures.

A further bottom-up factor potentially influencing the composition of the bacterial community is the present nutrient concentration. Pinhassi et al. (2004), Abell & Bowman (2005) as well as Grossart et al. (2005) were able to show that qualitatively different organic carbon from different algal exudates resulted in distinct bacterial communities. The distribution and concentrations of inorganic nutrients were also found to influence the structure of the bacterial assemblage (Hutchins et al. 2001, Pinhassi & Berman 2003, Pinhassi et al. 2006). Since it was also demonstrated that only a combined analysis of ambient temperatures and present nutrient concentrations allows a realistic assessment of their limiting effect on the bacterial community (Nedwell & Rutter 1994, Reay et al. 1999, Pomeroy & Wiebe 2001), the impact of both parameters was studied in a further experiment.

As the results of **chapter 3** show, genetic fingerprinting as well as the CARD-FISH analysis indicate that the given nutrient situation does have an influence on the extent of the temperature impact on the bacterial community structure. High nitrate concentrations apparently enhanced the impact of ambient temperatures which were detected for both bacterial fractions (not-attached and particle-attached) in this experiment, mainly during the degradation phase of the algal bloom. The above mentioned studies on the combined effect of nutrient situation and ambient temperatures on the bacterial community therefore confirmed.

Aside from the inherent limitations that working with man-made models of an ecosystem such as mesocosms entails, further restrictions that were to be expected regarding our experimental setting had to be faced. For one, the running time that was necessary to observe the experimental algal blooms from the beginning until the end of the degradation phase carried the risk of the development of a too pronounced wall growth which would have threatened to bias the pelagic signal of interest. It was therefore necessary to keep the light conditions at a level high enough to result in a satisfactorily fast bloom development to keep the running time of the experiment at a minimum. These light intensities exceeded natural early spring conditions at both of the conducted experiments. Further, organic material which is usually taken from the upper water layers by sedimentation remained was not removed from the tanks and thus still available for bacterial utilization. It is therefore evident that the experiments in this study did not allow a prognosis of developments taking place in the 'real world', but should be understood as possible effects of rising ambient temperatures on the basic mechanisms underlying the functional coupling of phyto- and bacterioplankton. All the above mentioned limitations notwithstanding, the experimental settings applied in this study were the only ones suitable for the analysis of the functional link between phyto- and bacterioplankton under defined temperature conditions.

Recapitulating, the presented work shows that an analysis of the elemental mechanisms at the basis of the coupling between marine algae and bacteria is possible by employing the above described mesocosm system set up in climatic chambers to simulate rising ambient temperatures. The results of this study furthermore imply that increasing temperature does affect the planktonic community as a whole and that the bacterial assemblage does respond in community structure to rising ambient temperatures. As the presented conclusions indicate, the composition of the particle-attached bacterial fraction showed a more distinct reaction to increasing water temperatures than the not-attached community. Moreover, the results on hand strongly suggest that the actual impact of increasing ambient temperatures on the marine bacterial community composition can only be reliably assessed if present inorganic nutrient concentrations are taken into account.

After having gained these first insights, further work in this area is certainly necessary to gain a more detailed picture on how future rising water temperatures might affect the community structure of marine bacteria. On the basis of the present results, a closer look at the particle-associated community in particular seems to be called for, as well as the use of more specific primers than those used in the study at hand. Screening both bacterial

assemblages with adequate specific primers, e.g. for the *Cytophga-Flavobacteria* or the *Roseobacter* group, and subsequent sequencing of bands of interest might be a first step towards a species specific identification of seemingly temperature-dependent taxa. Similar results could be achieved by creating clone libraries and ensuing sequencing of relevant clones. The design of probes specific for such organisms would then allow a targeted search for bacteria whose presence is apparently related to certain ambient temperatures. By applying molecular methods which link single-cell with functional analysis such as MICRO CARD-FISH (catalyzed reporter deposition fluorescence in situ hybridization combined with microautoradiography), it would even be possible to assign functional attributes to temperature-sensitive bacterial groups or species, permitting to further assess the consequences that shifts within the bacterial community structure caused by rising temperatures might have for the utilization of organic carbon in the oceans and therefore for the marine carbon cycle.

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Table of figures/tables

Introduction

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Chapter 2 – Temperature effects on the bacterial community composition during an algal spring bloom: a mesocosm study

Chapter 3 – Shifts in the bacterial community composition during an induced algal bloom under the influence of rising temperatures and different nutrient regimes

Erklärung

Die Kapitel 1-3 der vorliegenden Arbeit werden als Beiträge bei Fachzeitschriften eingereicht. Im Folgenden wird mein Beitrag an diesen zukünftigen Manuskripten erläutert:

- **Walther, K.**, Breithaupt, P., Lengfellner, K., Wohlers, J., Hoppe, H.-G., Sommer, U. und Jürgens, K. (2008) The effects of rising temperatures on a planktonic community during an induced phytoplankton spring bloom (In Vorbereitung zur Einreichung bei Aquat. Microb. Ecol.)

Planung, Durchführung und Beprobung des Mesokosmosexperiments; Durchführung der PCR, DGGE, Clusteranalyse sowie Identifizierung der Sequenzen für Eu- und Prokaryoten; quantitative Bestimmung der Nanoflagellaten und virusähnlichen Partikel; Durchführung der CTC-Fixierungen und Auswertung der entsprechenden FCM-Zählungen; Erstellen der Erstfassungen des Manuskripts; Korrektur durch Prof. Klaus Jürgens.

- **Walther, K.**, Breithaupt, P., Lengfellner, K., Wohlers, J., Hoppe, H.-G., Sommer, U. und Jürgens, K. (2008) Temperature effects on the bacterial community composition during an algal spring bloom: a mesocosm study (In Vorbereitung zum Einreichen bei Aquat. Microb. Ecol.)

Planung, Durchführung und Beprobung des Mesokosmosexperiments, Durchführung der RNA-Extraktion, PCR, DGGE, Clusteranalyse sowie Identifizierung der bakteriellen Sequenzen; Durchführung der CARD-FISH-Analyse; Erstellung der Erstfassungen des Manuskripts; Korrektur durch Prof. Klaus Jürgens.

- **Walther, K.**, Breithaupt, P., Wohlers, J., Riebesell, U. (2008) Shifts in the bacterial community composition during an algal bloom under the influence of rising temperatures and different nutrient regimes (In Vorbereitung zur Einreichung bei Aquat. Microb. Ecol.)

Planung der Durchführung und Beprobung des Experiments; Durchführung der RNA-Extraktion, PCR, DGGE und Clusteranalyse; Durchführung der CARD-FISH-Analyse; Erstellen der Erstfassung des Manuskripts; Korrektur durch Prof. Ulf Riebesell.

Publikationsliste

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Hoppe, H.-G., Breithaupt, P., **Walther, K.**, Koppe, G., Bleck, S., Sommer, U., Jürgens, K. 2008. Climate warming in winter affects the coupling between phytoplankton and bacteria during the spring bloom: a mesocosm study. Aquatic Microbial Ecology 51: 105-115

Tagungsbeiträge

Vorträge

Walther, K., Bleck, S., Hoppe, H.-G., Jürgens K. 2005. The influence of different temperatures and phytoplankton communities on bacterial activity and composition. 1. Annual Meeting AQUASHIFT, 29. September, Kiel, Deutschland.

Walther, K., Breithaupt, P., Sommer, U., Hoppe, H.-G., Jürgens, K. 2006. How do different temperature regimes affect bacterial activity and community composotion during an algal spring bloom? 2. Annual Meeting AQUASHIFT, 14. November, Kiel, Deutschland.

Walther, K., Breithaupt, P., Sommer, U., Hoppe, H.-G., Jürgens, K. 2006. Bacterial community composition along a temperature gradient during an algal spring bloom – a mesocosm study. Baltic Sea Science Conference, 20. März, Rostock, Deutschland.

Walther, K., Breithaupt, P., Sommer, U., Hoppe, H.-G., Jürgens, K. 2007. Bacterial community composition along a temperature gradient during an algal spring bloom – a mesocosm study. 42nd European Marine Biology Symposium, 27. August, Kiel, Deutschland.

Walther, K., Breithaupt, P., Sommer, U., Hoppe, H.-G., Jürgens, K. 2007. Bacterial community structure and cell-specific activity along a temperature gradient during an algal spring bloom. 37th Annual Conference of the Ecological Society of Germany, Switzerland and Austria (GfÖ), 12. September, Marburg, Deutschland

Posterbeiträge

Walther, K., Sommer, U., Hoppe, H.-G., Jürgens, K. 2006. Composition of the bacterial community in a temperature-modulated mesocosm system during an algal spring bloom. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM)19.-22. März, Jena, Deutschland.

Walther, K., Sommer, U., Hoppe, H.-G., Jürgens, K. 2006. Bacterial community structure and cell-specific activity along a temperature gradient during an algal spring bloom. 2. Annual Meeting AQUASHIFT, 14.-16. November, Kiel, Deutschland.

Walther, K., Sommer, U., Hoppe, H.-G., Jürgens, K. 2006. Composition of the bacterial community in a temperature-modulated mesocosm system during an algal spring bloom. 11th International Symposium on Microbial Ecology (ISME), 20.-25. August, Wien, Österreich.

Vorträge als geladene Sprecherin

Walther, K. 2007. Die bakterielle Gemeinschaftsstruktur unter dem Einfluss erhöhter Frühjahrstemperatur, 22.November 2007 vor der Abteilung Marine Mikrobiologie am IFM-GEOMAR, Kiel

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